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An Unsuspected Source of Error in Studies of Particle Transport by Lamellibranch Gill Cilia[1]

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In appraising physiological and pharmacological reactions of cilia, no method has been so widely employed as the measurement of the velocity at which inert particles are moved across ciliated mucous membrane. The classical studies of Engelmann (1879), Lommel ('08), Gebhardt ('09), and Hill ('28) all relied heavily on observations of particle transport. A remarkable variety of particles has been employed. The nature of the granule is thought to be unimportant providing that its insolubility renders it chemically inert and its density is so low that it can be carried along in the currents of fluid generated by the active cilia. Using isolated mammalian trachea, Hill ('28) reported that he was able to obtain satisfactory data with the following assortment of test particles: charcoal, iron and aluminum fillings, zinc oxide, mercurous chloride, barium carbonate, and bismuth carbonate.

In studies with particles of a single type, the rate of transport did not appear to be critically dependent upon the particle size, but to obviate any subtle influences of size and shape, several investigators have restricted their observations to single objects. A disc with a diameter of several millimeters has often been used, in order to insure that no small area of atypical activity would severely prejudice the results. For example, most of Gray's data ('23, '24) were obtained with a "very small circular plate of platinum" which moved along the submerged ciliated mucous membrane of *Mytilus* gill. The same theoretical advantages can be obtained by inverting the mucous membrane and measuring how fast it crawls along the flat bottom of the container in which it is submerged (Nomura and Tomita, '33; Weller and Ronkin, '52; Tomita, '55).

In a recent study of lamellibranch gill cilia in this laboratory, we were puzzled by the apparent failure of serotonin (5-hydroxytryptamine) to stimulate the activity of frontal cilia, as appraised by the method of particle transport, in view of its potent cilio-accelerator action on lateral and abfrontal cilia, as appraised by stroboscopic and cinematographic methods (Gosselin, '61). A detailed study of this phenomenon revealed that the movement of small particles along the frontal surface was accelerated by serotonin, while the transport of large particles, even of the same material, was distinctly inhibited. Since we now can trace the cause of this anomalous reaction to the peculiar structure of the gill, it is not likely to be a difficulty in studies with other mucous membranes. This unrecognized source of error is important, however, because it may well have influenced results in earlier studies of gill cilia, results upon which are based so many widely held generalizations about the physiological and pharmacological behavior of cilia.

MATERIALS AND METHODS

Sources, technics of storage, and methods of preparation of *Mytilus edulis* and *Modiolus demissus* have been described previously (Gosselin, '61). From the excised gill was teased an entire double lamellae (White, '57). The method of mounting it was crucial to a successful study of the frontal cilia. A small block of paraffin was sealed on the bottom of a Petri dish, which in turn was suspended in a larger vessel of tap water to serve as a constant temperature bath. The bath temperature was maintained by a glass coil through which was pumped water from a thermostated and refrigerated reservoir. Most experiments were performed at 20 or 21°C.

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The portion of gill chosen for study was mounted on a paraffin block so that the top surface, composed of the frontal faces of adjacent filaments, was submerged in sea water at a depth of several millimeters. It proved to be difficult to anchor the preparation without disturbing the normal anatomical relationships of the filaments to one another. Without immobilization the action of the frontal cilia caused the array of filaments to crawl in such a direction that the distal or free margin of the gill trailed. The proximal margin (by which the intact gill joins the visceral mass) was fenced in successfully by inserting dissecting pins into the paraffin block. The anterior and posterior ends of the gill plate were anchored with small lead discs, but every attempt to hold the preparation by weighting all of the filaments caused them to separate from one another and settle with their lateral surfaces uppermost instead of their frontal facets. Considerable care was exercised to avoid touching the proximal margin or root of the excised gill plate, which is occupied by a prominent blood vessel that feeds each filament (afferent branchial vessel). Even gentle manipulation of this vein produced a contraction ring which lasted for several minutes and caused the gill plate to buckle. In a satisfactory preparation the central filaments lay flat, allowing repeated observations of particle transport for periods of many hours.

While the gill plate was kept under observation through a dissecting scope equipped with an ocular micrometer, a test particle was allowed to settle on or near the proximal gill margin. Currents generated by the frontal cilia carried it to the distal margin. As it moved along parallel to the axis of the filaments, its velocity was measured over a fixed course, 4 to 6 mm long, coinciding with the middle segment of the centrally located filaments. The particle was then returned to the starting position and the transit time was remeasured. Each datum used in the statistical analysis was an average of three such observations made within a period of two minutes. This sequence of observations was repeated at intervals of two to 5 minutes.

Artificial sea water at pH 7.8 (MBL formula no. 4) was used for all serotonin (5-HT) solutions. Concentrations of 5-HT refer to the creatinine sulfate salt in gm/ml. In order to expose the gill to different concentrations of this amine, gentle suction with a syringe was used to remove most of the 20 ml of sea water in which the tissue was immersed. When this fluid was replaced carefully with an equal volume of solution, the gill filaments resumed their former orientation as judged by observations of particle transport in appropriate control studies. As a method of terminating an exposure to 5-HT, this procedure was of course inefficient, and several changes of sea water were usually required. Every preparation was exposed to each 5-HT concentration for a period of at least 20 and usually 30 to 40 minutes. Each tabulated datum refers to the average velocity observed during an entire test period, except that observations during the first 5 or even 10 minutes were discarded whenever they revealed a progressive shift in ciliary activity during this period of equilibration.

RESULTS

Various kinds of particles were tested on the lamellibranch gill, notably aluminum dust, lycopodium seeds, fragments of glass wool, beach sand, pure ground quartz, and poppy seeds. Several of these materials were unsatisfactory only because they clumped when suspended in sea water. Poppy seeds proved to be the easiest to use. For each experiment a single seed was selected, usually with dimensions of about 1 mm and a weight of less than 1 mg. Soft seeds and those that floated were rejected. At 20°C the frontal cilia of *Mytilus* and *Modiolus* gills moved poppy seeds with an absolute velocity of 0.2 to 0.6 mm/sec. As long as the frontal surface of the gill was submerged at least 2 mm below the surface, the depth was of no importance in terms of the transit velocity. For reasons outlined by Hill ('57), shallower solutions were not tested.

Figure 1 depicts variations in the rate at which *Mytilus* frontal cilia moved a poppy seed under the influence of various concentrations of 5-HT. Two periods of exposure to unmodified sea water were included. Serotonin concentrations of 10^{-9}, 10^{-8}, and 10^{-7} gm/ml in sea water appear to have retarded the poppy seed, but the effect was not large until a concentration of 10^{-6} was used. This action of 5-HT was readily reversed by substituting plain sea water; in many experiments it did not disappear so promptly.

Similar results were obtained with other poppy seeds and other preparations of *Mytilus* and *Modiolus* gill, as summarized in table 1. Each number represents a difference between the mean velocity with and without added 5-HT, expressed as a percentage of the mean control rate. In experiments with several control periods, the nearest was chosen as the basis of comparison. Small numbers in parentheses indicate 0.95 confidence limits of each estimate, according to a modified t test. Values significantly different from zero are set in italic. As indicated by the negative signs, every significant change that was observed in the transit velocity was a decrement. This inhibitory reaction appeared to be dose-dependent. In terms of the minimal concentration required to produce a significant effect, *Mytilus* gill proved to be more sensitive than *Modiolus* to the action of 5-HT, but a concentration of 10^{-6} invariably produced a significant decrement in velocity amounting to as much as 50%. Qualitatively similar observations were made on a few specimens of a fresh-water mussel (*Anodonta spp.*).

When a suspension of ground quartz was substituted for the poppy seed, a different response was encountered, as seen in figure 2. This suspension of crystalline quartz consisted of irregularly shaped particles with dimensions varying between 10 and 50 μ. Within this range of sizes, all particles moved with essentially the same velocity, which generally was a little faster than that of a poppy seed. In this series of measurements no attempt was made to use and then recover a single quartz particle. Instead one drop of a dilute suspension was applied to the proximal margin of the gill just before each observation, and the group velocity was then measured. As before each point represents the average of three trials. Exposure to 5-HT caused quartz particles to move faster (fig. 2). This response too was graded and reversible.

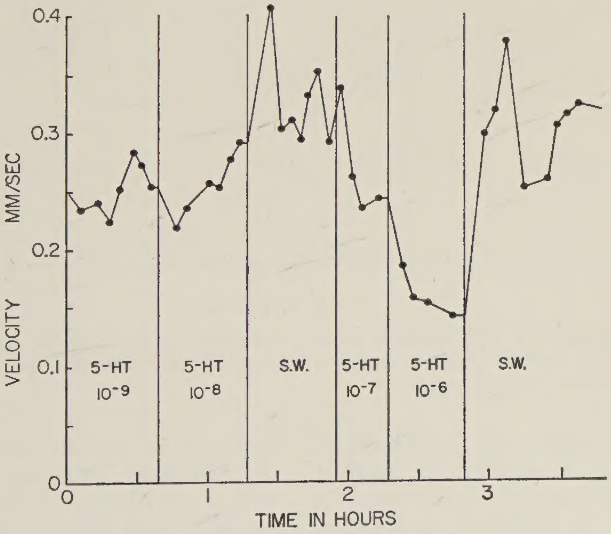

Fig. 1 The velocity at which a poppy seed is transported by the activity of frontal cilia on *Mytilus* gill in the presence of various concentrations (gm/ml) of exogenous serotonin (5-HT). S.W. indicates sea water without drug.

TABLE 1

Transport of poppy seeds by frontal gill cilia.[1] *Shown as change in velocity (in per cent of control)*[2]

5-HT concentration[3]			
10^{-9}	10^{-8}	10^{-7}	10^{-6}
		Mytilus	
	−20 (±8)	*−30* (±3)	
−1 (±10)	*−26* (±7)	*−28* (±7)	
−9 (±7)	*−21* (±12)	−20 (±14)	*−48* (±8)
2 (±12)		*−18* (±9)	
	3 (±6)	2 (±5)	*−20* (±6)
			−45 (±8)
			−22 (±6)
		Modiolus	
	−4 (±12)	−4 (±8)	
	11 (±16)	8 (±9)	*−15* (±11)
		−19 (±5)	*−30* (±6)
		−27 (±10)	
	−16 (±11)	3 (±13)	

[1] Each line of data refers to a different preparation.

[2] Italic type designates those means which differ significantly from zero at the 0.05 level of significance according to a two-sided t test modified for analyzing ratios. In this analysis the estimated standard error of the ratio $\left(\frac{\text{mean vel. }(\overline{x})\text{ with 5-HT}}{\text{mean vel. }(\overline{y})\text{ without 5-HT}} - 1\right)$ is given by: $\text{s.e. of ratio} = \frac{1}{\overline{y}}\sqrt{(\text{s.e. of }\overline{x})^2 + \frac{(\text{s.e. of }\overline{y})^2\overline{x}^2}{\overline{y}^2}}$

[3] Milligrams serotonin creatinine sulfate per milliliter sea water.

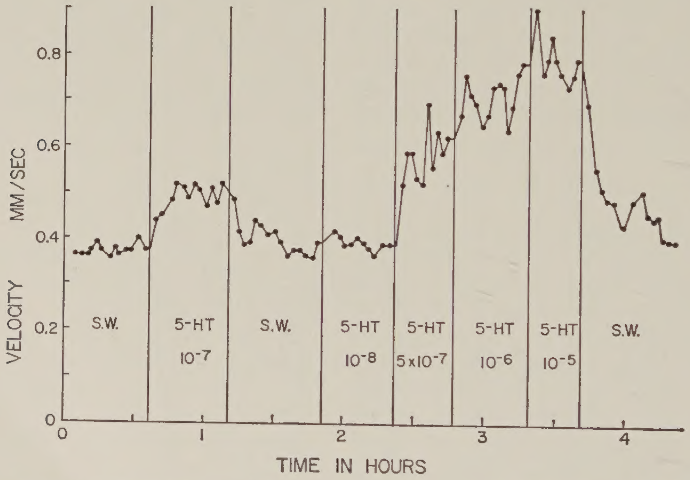

Fig. 2 The velocity at which small quartz particles are transported by the frontal cilia of *Modiolus* gill in the presence of various concentrations (gm/ml) of serotonin (5-HT). S.W. indicates sea water without drug.

Table 2 summarized all experiments with quartz particles applied to *Mytilus* and *Modiolus* gill. Rates and fiducial limits have the same meaning as in table 1. Again statistically significant changes are recorded in italic. In this series of experiments the only response to 5-HT was an increase in transit velocity; no examples of inhibition were encountered. The reaction of 5-HT at a concentration of 10^{-6} gm/ml was highly significant in every case; in some instances the rate more than doubled. At 10^{-7} gm/ml the increment was significant 4 out of 5 times with

Mytilus gill and two out of 5 times with *Modiolus* gill. That *Mytilus* was more sensitive is also evidenced by the failure of *Modiolus* gill to respond to concentrations below 10^{-7}, while *Mytilus* reacted significantly three out of 5 times.

In an attempt to explain the contradictory actions of 5-HT on the transport of poppy seeds and crushed quartz, several kinds of experiments were performed. That 5-HT does not cause a toxic or inhibitory substance to leach out of poppy seeds

TABLE 2

Transport of ground quartz by frontal gill cilia.[1] *Shown as change in velocity (in per cent of control)*[2]

5-HT concentration[3]					
10^{-8}	5×10^{-8}	10^{-7}	5×10^{-7}	10^{-6}	10^{-5}
		Mytilus			
4 (±8)		*57* (±18)		*103* (±20)	
25 (±9)	*34* (±9)	*74* (±12)		*142* (±18)	
		21 (±20)			
32 (±17)		*73* (±8)		*124* (±13)	
				53 (±21)	
6 (±10)		7 (±10)		*49* (±16)	
		Modiolus			
1 (±13)		2 (±13)		*14* (±10)	
		34 (±4)		*80* (±11)	77 (±11)
	8 (±12)	2 (±10)		*32* (±25)	
		3 (±5)	*21* (±7)	35 (±7)	*42* (±7)
1 (±4)		*34* (±4)	*48* (±10)	76 (±10)	*92* (±9)

[1] Each line of data refers to a different preparation.

[2] See footnote 2, table 1.

[3] Milligrams serotonin creatinine sulfate per milliliter sea water.

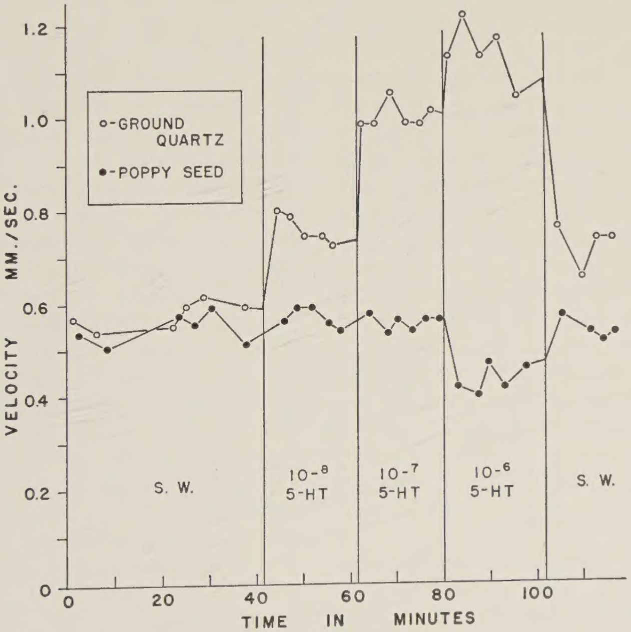

Fig. 3 The velocities at which small quartz particles and a poppy seed are transported by *Mytilus* frontal cilia in the presence of various concentrations of 5-HT. S.W. indicates sea water without drug.

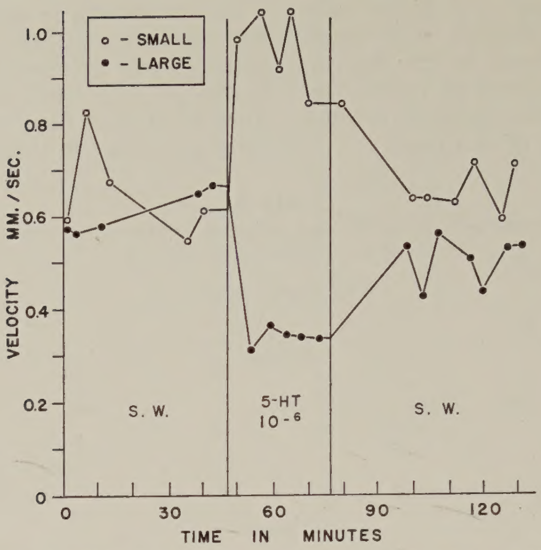

Fig. 4 The ability of exogenous 5-HT to enhance the transit velocity of small quartz particles and simultaneously to depress the velocity of a large quartz grain on *Mytilus* gill. S.W. indicates sea water without drug.

is demonstrated by tests in which rates of transport of seeds and of quartz were measured alternately on the same excised gill. As seen in figure 3, 5-HT at a concentration of 10^{-6} markedly accelerated small quartz particles at the same time that the movement of a poppy seed was retarded. Indeed several of the experiments in tables 1 and 2 represent such combined exposures.

That the poppy seed was as chemically inert as quartz in this system is indicated by experiments in which a large quartz particle was substituted for the seed. This irregular grain of sand was clear and colorless; it was about the same size as the average poppy seed (1.5 × 0.9 × 0.9 mm), but it weighed more (1.3 mg). Like the poppy seed its motion was retarded by 5-HT at the same concentration and at the same time that small particles of quartz were accelerated (fig. 4). Both of these effects were highly significant.

DISCUSSION

Because both the 5-HT-induced retardation of poppy seeds and acceleration of quartz granules were statistically significant, graded with respect to the 5-HT concentration, and completely reversible, the question arises: what, if anything, do these observations indicate about the activity of frontal gill cilia? An explanation of this dilemma can be found only when one relates differences between the particles to the peculiar structure of the gill.

Both poppy seeds and crushed quartz are believed to be chemically inert on molluscan gill, and both have been used successfully by others (Kordik et al., '52; Hill, '28) for appraising the activity of various ciliated tissues. That neither class of particles exerted a persistent excitatory or inhibitory action on ciliary movement is indicated by experiments in which the gill was exposed alternately to quartz and to poppy seeds without interfering with the characteristic effect of 5-HT on the transit velocity of either (e.g., fig. 3). The density difference between quartz and poppy seeds appears to have been irrelevant since a quartz particle having the same dimensions as a poppy seed reacted like it by moving more slowly in the presence of 5-HT. Size is believed to have been the only pertinent difference between the 5-HT-accelerated granules of crushed quartz and the 5-HT-retarded grain of beach sand.

No one seems to have asserted categorically that the velocity of particle transport over ciliated mucous membrane is independent of particle size, but this independence is implicit in many earlier studies. At least all small particles of sufficiently low density are believed to behave as equivalent inert markers of the moving film of mucus in which they become imbedded. This predominant opinion is entirely in accord with the present observation that all particles of crushed quartz with diameters between 10 and 50 μ traveled at the same velocity on the frontal face of lamellibranch gill filaments. With larger particles the published evidence is not so convincing. Probably various mechanical influences arise to complicate the transport process. In the diameter range of 1 to 4 mm, Stewart ('48) showed that the transit velocity of metal discs across the excised frog palate is a function of the contact pressure but not of the contact area. Although many investigators have undoubtedly encountered subtle differences in the ability of ciliated epithelium to handle particles of different sizes, this report is believed to be the first demonstration of a qualitative difference. Since no comparable phenomenon has been reported for other mucous membranes, the ultimate explanation probably lies in the unique structure of the gill.

Gill cilia generate two important currents of fluid near the frontal edge of each filament. The true frontal cilia (FC in fig. 5) direct particles along the frontal face to the food groove at the free margin of the gill, i.e., in a direction perpendicular to the plane of figure 5. At the same time the powerful lateral cilia (LC) are serving to suck fluid into the spaces between adjacent filaments and thence to the abfrontal surface (viz., arrows of fig. 5). These two fluid currents are directed perpendicularly to each other. To the extent that fluid movement in the vicinity of the gill is laminar, one does not expect a very small particle on or near the frontal face (A in fig. 5) to be influenced by currents which the lateral cilia generate. Of course if such a particle wanders far enough off course, it becomes

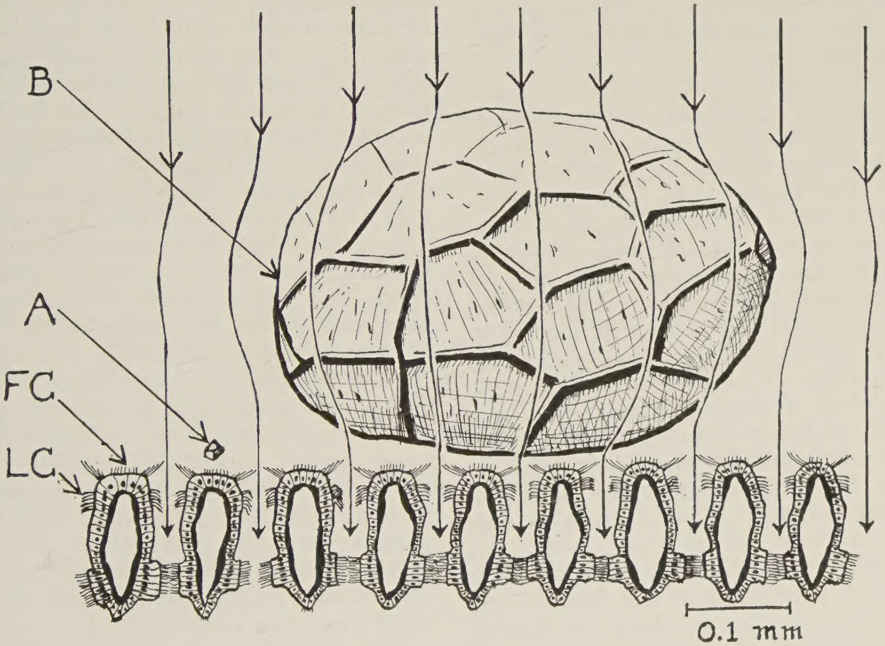

Fig. 5 A schematic cross-sectional representation of adjacent filaments of the *Mytilus* gill, depicting frontal cilia (F.C.) and lateral cilia (L.C), and demonstrating the relationship of a small quartz particle (A) and a poppy seed (B) to currents generated by the lateral cilia. In relation to the scale, A is somewhat larger and B somewhat smaller than the typical particles used in this study.

immediately trapped in the lateral stream and flushed down between the filaments. This phenomenon was observed repeatedly during these studies, especially when the gill was exposed to 5-HT. Obviously no useful measurements could be made on particles which suffered this fate, and so these episodes did not prejudice the transit velocities reported here.

On the other hand large particles on the frontal face (B in fig. 5) could not escape the conflicting forces that arose from these two currents of fluid. The average poppy seed spanned almost a dozen filaments of the *Mytilus* gill plate and even a larger number of the slenderer filaments of *Modiolus*. As poppy seeds and other relatively large objects were carried along the gill plate by the activity of the frontal cilia, they were forced to hug the frontal surface by the vertically directed stream of sea water. When the stimulatory action of 5-HT on the lateral cilia (Aiello, ’60; Gosselin, ’61) caused this vertical current to become sufficiently intense, the poppy seed was pressed against the frontal surfaces so tightly that propulsion by the frontal cilia was impaired, in spite of their more vigorous beat.

According to this explanation, to which no acceptable alternatives have been found, stimulation is the genuine response of frontal cilia to 5-HT, in accord with table 2. The transport of large particles, however, is interfered with by the activity of the lateral cilia which are excited by essentially the same concentrations of 5-HT that stimulate the frontals (Gosselin, ’61). A seasonal fluctuation in the sensitivity of lateral cilia is revealed by the concentrations of exogenous 5-HT necessary to elicit a maximal response (Gosselin, ’61). Current observations on frontal cilia are not extensive enough to indicate whether there are comparable shifts in their dose-response curve. At least seasonal variations are not responsible for those differences which we now relate to the influence of particle size because table 1, for example, is based on experiments performed in the summer and fall of 1959 as well as the spring of 1960.

Implicit in the explanation proposed here is the existence of a retarding force on large particles resting on any gill plate whether chemically stimulated or not. During drug-free periods transit velocities of large and small particles were often the same (e.g., figs. 3 and 4), but by pooling all control observations, ground quartz particles were found to have moved faster than poppy seeds. Although the mean velocities proved to be significantly different at the 0.01% level (0.42 vs. 0.32 mm/sec.), the difference in many experiments was unimpressive. Apparently the cilioacceleratory action of 5-HT was required to unmask this retarding force by intensifying the lateral stream.

We conclude that the puzzle illustrated in figures 3 and 4 has a disappointingly trivial solution. At least the explanation as outlined above cannot be generalized to other ciliated mucous membranes, and in accordance with this expectation we have been unable to detect a comparable phenomenon with the excised frog palate or the mammalian trachea. A far from trivial consequence of this solution is the grave question it raises about the validity of conclusions already in the literature. If experiments with large particles can make a potent cilioexcitatory substance like 5-HT look like an inhibitor, the same phenomenon may have misled Gray (’24) in his studies on the influence of pH on frontal gill cilia or may have distorted the temperature relationship he described (’23). Because the same complications may arise when ciliary activity is evaluated by measuring how fast excised pieces of gill are able to crawl along the bottom of a glass vessel, the alleged ciliary effects of hypotonicity (Hopkins, ’49; Tomita, ’55), of dinitrophenol (Weller and Ronkin, ’52), and of various drugs and poisons (Nomura ’37; Pritchard and Giese, ’50) require further investigation. Since the activity of frontal cilia cannot be appraised satisfactorily by stroboscopic or cinematographic methods, observations on particle transport must suffice. This report demonstrates that only small particles should be used in such studies.

SUMMARY

1. The frontal cilia on the excised gills of *Mytilus edulis* and of *Modiolus demissus* move small particles of crushed quartz

slightly more rapidly than poppy seeds or large quartz particles.

2. Under the influence of exogenous serotonin (5-HT) small particles are transported significantly more rapidly and large particles significantly more slowly than during control periods without 5-HT. Both effects proved to be graded with respect to the 5-HT concentration and completely reversible at all concentrations.

3. By ruling out various plausible solutions of this paradox, an acceptable explanation was found in terms of the peculiar structure of the gill. When a particle which is large relative to the thickness of a gill filament is carried along the frontal surface by the activity of the frontal cilia, its motion is retarded by a perpendicular current of sea water which is generated by the neighboring lateral cilia. This retardation becomes much more evident when the lateral cilia are stimulated by exogenous 5-HT.

4. Particles which are small relative to the thickness of a gill filament escape the influence of the lateral stream and move along the frontal face more rapidly in the presence of 5-HT.

5. Thus stimulation is the genuine response of both frontal and lateral cilia to 5-HT.

6. Because of the spurious effects which they display, large particles should not be used for estimating the activity of gill cilia.

ACKNOWLEDGMENTS

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Exogenous Enzymes of *Neurospora* Conidia and Mycelia

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The exoenzymes of fungi may be conveniently divided into 4 main classes. The first class includes enzymes liberated from spores upon contact with water and is illustrated by the diphosphopyridine nucleotidase of *Neurospora crassa*, (Zalokar and Cochrane, '56). The second group is exemplified in *Myrothecium verrucaria*, (Mandels, '51, '56), where invertase is released from spores in the course of germination and early growth. The third group includes enzymes liberated from mycelia during the linear phase of growth, while the 4th class of enzyme is liberated by older mycelia—apparently in the course of cell lysis, (Reese and Mandels, '59). Classes 3 and 4 have been studied extensively in fungi, although relatively few studies have critically distinguished these two groupings.

The purpose of this paper is to report the exogenous occurrence of several enzymes of *Neurospora* conidia. Special emphasis has been placed upon the changes that occur in β-aryl glucosidase during the life cycle of *Neurospora*, as one of the little understood first class of enzymes.

MATERIALS AND METHODS

1. *Organism and media*

Cultures of *Neurospora crassa* were grown on glycerol complete medium containing minimal medium, (Beadle and Tatum, '45), plus Bacto-casitone (Difco) 1 gm, yeast extract (Difco) 25 gm, agar (Difco) 15 gm, glycerol (Baker Chem. Co.) 8 ml, and vitamin stock solution 10 ml per 1000 ml of medium.

2. *Harvest of dry conidia*

Conidia were harvested by shaking fully conidiated culture tubes and collecting the air borne spores on a cotton filter in a pyrex tube (8 mm × 150 mm) with a stream of air created by a water driven aspirator. Conidia were removed from the filter by a flow of distilled water and allowed to stand in 5 ml of water (usually 10 minutes) and were centrifuged in a Sorvall angle centrifuge type SP/X for 20 minutes at 3000 g. This water extract (supernatant solution) was then tested for enzyme activity. The conidial pellet was dried at 85°C to constant weight.

3. *Enzyme preparations from air-dried mycelia*

Mycelia were harvested at various times and dried on a plate glass surface in a stream of air. The dry mycelia were weighted and ground in a device similar to that of Landman and Bonner, ('52). Powdered mycelia (100 mg) were suspended in 5 ml of distilled water and incubated for 20 minutes at 25° then quickly frozen. After 12 hours the suspension was thawed and centrifuged at 8000 g for 30 minutes.

The β-glucosidase assay was based on the use of the *p*-nitrophenyl-β-D-glucopyranoside (California Corporation for Biochemical Research) as a chromogenic substrate. The reaction was followed by the release of nitrophenol (Aizawa, '39) and was measured at a wave length of 410 mμ in a Beckman D.U. spectrophotometer or in a Spectronic 20 spectrophotometer equipped with a roto-cell. Both spectrophotometers were equipped with constant temperature jackets adjusted to 37°C. The *continuous method* of assay was run in a cuvette in a final volume of 1 ml buffered with 0.1 M potassium phosphate at pH 7.1. The reaction was started by the addition of 0.1 ml enzyme solution to 0.9 ml of buffer containing 1 mg of the substrate. The control cuvette contained buffered substrate plus 0.1 ml distilled water. In the *discontinuous method* of β-glucosidase determination, 0.1 ml of enzyme solution was pipetted into a 10 × 75 mm test tube. After two minutes of incubation in a water bath at 37°C the reaction was started by the addition of 0.9 ml of buffer containing 1.0 mg of substrate. The buffer was 0.1 M citrate-phosphate at pH 5.0, to be near the optimum pH for *Neurospora* β-glucosidase. The reaction was stopped by the addition of 0.5 ml of 0.1 M tris buffer (tris hydroxymethyl amino-methane). This buffer also brought the nitrophenol into its optimal pH range (about pH 8) for maximum absorption at 410 mμ. Alkaline phosphatase was determined by a modification of the method in Sigma Chemical Company (Technical Bulletin 104, '55). The chromogenic substrate was *p*-nitrophenyl phosphate (Sigma Co., St. Louis, Mo.) and the release of nitrophenol was measured at 410 mμ in a 1 ml volume containing 1 mg of substrate and 0.1 M tris buffer at pH 8.6. The reaction temperature was 37°C. Paper chromatography was used to identify carbohydrases in water extracts of conidia. Each prospective substrate was first incubated with conidial extract in the following system: Citrate-phosphate buffer pH 5, 0.01 M, 0.25 ml; conidial wash, 0.25 ml; a 1% solution of the substrate, 0.25 ml. Incubation was at 37°C, and the reaction was stopped by quickly drying 10 μl of the reaction mixture on Whatman no. 1 chromatographic paper. The enzyme substrate and product(s) were separated by ascending chromatography with a solvent system of n-butanol, pyridine and water (6:4:3, v/v). The chromatogram was developed for 12 hours and then dried in a current of air followed by a dip in a reagent containing Benzidine in acetone (Harris et al., '54) to detect the sugars. Disaccharides were purchased from the California Corporation for Biochemical Research.

RESULTS AND COMMENTS

The conidia of several *Neurospora* strains were examined for exoenzymatic activity. The enzymes selected were β-glucosidase, β-galactosidase and alkaline phosphatase so that the identical steps involved in the assay of these enzymes would greatly simplify the assay procedure. Conidia were collected by an air filter from cultures grown 8 days at 25°C, and then suspended in distilled water to extract exoenzymes. A microscopic examination of this conidial suspension did not show any mycelial contamination. When the conidia were removed by centrifugation, this water extract contained relatively high levels of β-glucosidase and somewhat lower and more variable levels of alkaline phosphatase (table 1). These preparations failed to show β-galactosidase by the method of Landman and Bonner ('52). The difference in the enzyme activities of the extracts of these strains may be explained by the difference in physiological age of the wild type and thiamineless cultures at the time of harvest (Eberhart and Tatum, '61). Tests of conidia from other *thi-1* and wild type strains showed no consistent association of β-glucosidase or alkaline phosphatase levels with the *thi-1* gene.

An exhaustive removal of β-glucosidase from wild type conidia was studied in an experiment involving a series of centrifugial washings with distilled water (table 2). The supernatant solution was removed and analyzed after each centrifugation. After 4 identical washings the co-

TABLE 1

Enzyme activities in water extracts of Neurospora conidia[1]

Strain	β-Glucosidase[2]	Alkaline phosphatase[3]
56501–3–90 (*thi-1*)	0.225	0.105
56501–3–88 (*thi-1,lo*)	0.200	0.011
SY7A (wild)	0.161	0.019
SY4a (wild)	0.134	0.009

[1] Cultures were grown 8 days at 25°C on glycerol complete agar slants. Conidia were collected with an air filter and then washed with water by centrifugation. The supernatant solution was the source of enzyme activity. (See Materials and Methods.)

[2] Continuous method: values are Δ O.D./10 min./mg dry conidia.

[3] Continuous method: values are Δ O.D./10 min./mg dry conidia.

TABLE 2

Removal of β-glucosidase from Neurospora conidia by water and acetone washing[1]

Centrifugation no.	β-Glucosidase[2]
1	0.448
2	0.068
3	0.004
4	0.006
5[3]	0.070

[1] The *Neurospora* wild type strain SY4a was grown 6 days on glycerol-complete agar media. Conidia were collected with an air filter, washed by 4 successive centrifugations in water, treated with cold acetone and washed finally by centrifugation in water. The supernatant solutions were the sources of β-glucosidase activity. (See Materials and Methods.)

[2] Discontinuous method: values are Δ O.D./10 min./mg dry conidia.

[3] After acetone treatment.

nidia were treated with cold acetone and then washed with water again (see Materials and Methods). Most of the β-glucosidase activity was removed in the first extraction, thus demonstrating the exogenous nature of this enzyme. The release of a significant amount of β-glucosidase after acetone treatment suggests that the conidia possessed a residual or endo-β-glucosidase that was released when the surface properties of the cell were altered by acetone treatment. This residual activity was small compared to the total β-glucosidase removed from the conidia.

Alkaline phosphatase was examined in addition to β-glucosidase in a similar experiment (table 3). Conidia of a wild type strain were extracted with water, treated with cold acetone and finally extracted with water again. A relatively high amount of alkaline phosphatase activity was released after the acetone treatment. This suggests that alkaline phosphatase is primarily endogenous and is presumably located either on or within the cell surface of the conidia.

Beta-galactosidase activity was absent in preparations of acetone treated conidia. In experiments where toluene was used to disrupt conidia the release of high levels of β-galactosidase as well as alkaline phosphatase and β-glucosidase was obtained. This may indicate either a differential inactivation of β-galactosidase by toluene and acetone or the location of β-galactosidase and alkaline phosphatase on different cell structures.

Since β-glucosidase was very active in conidial extracts other carbohydrases were investigated in similar preparations from wild type strain SY7A. Different disaccharides were incubated with conidial water extract (see Materials and Methods). Samples were periodically removed from the reaction mixture and then chromatographed on paper to separate the enzyme products. This procedure was used to give a general test system for this class of enzymes that would be simple yet unambiguous in following substrate disappearance while also following product formation. The results of three such experiments are shown in table 4. They clearly establish the presence of maltase, cellobiose, and invertase in the conidial extracts by the appearance of glucose during incubation. This is correlated with the disappearance of the various substrates with time. It is not known as yet if cellobiase activity in this preparation is identical to the β-glucosidase measured by the hydrolysis of *p*-nitrophenyl-β-D glucopyranoside. Similar experiments with cellulase (carboxymethyl cellulose) and β-galactosidase (lactase) showed no enzyme activity in conidial ex-

TABLE 3

Differential removal of β-glucosidase and alkaline phosphatase from Neurospora conidia[1]

Activity in supernatant solution of:	β-Glucosidase[2]	Alkaline phosphatase[3]
Centrifugation no. 1	0.157	0.017
Centrifugation no. 2[4]	0.105	0.508

[1] Conidia were from a culture of strain SY7A (wild) grown 7 days at 25°C on a glycerol complete slant. Conidia were harvested by filtration from air, washed with water and centrifuged, treated with acetone and finally washed with water and centrifuged again. The supernatant solutions were the sources of enzyme activity.

[2] Continuous method: values are: Δ O.D./10 min./mg conidia.

[3] Continuous method: values are: Δ O.D./10 min./mg conidia.

[4] Following acetone treatment.

TABLE 4

Enzyme activities in water extracts of conidia as determined by chromatographic identification of the enzyme products

Enzyme	(a.) Substrate (b.) Product	Relative concentration of substrates and products[1]			
		0	30 min.	1 hr.	6 hr.
Maltase	a. Maltose	+++++	++++	+++	+
	b. Glucose	—	+	++	+++++
Cellobiase	a. Cellobiose	+++++	++++	+++	trace
	b. Glucose	—	+	++	+++++
Invertase	a. Sucrose	—	(not visible with Benzidine indicator)		
	b. Glucose	—	+	++	++++
	c. Fructose	—	+	++	++++

[1] Carbohydrates were separated with a n-butanol, pyridine, water (6:4:3 v/v) solvent system and then detected after a dip in benzidine-acetone reagent.

tracts. The observation that lactose was not attacked by the conidial extract correlates with the inability of similar preparations to act on o-nitrophenyl-β-D galactoside.

The increase in β-glucosidase in growing *Neurospora* mycelia was studied in aerated cultures. Conidia of wild type SY4a were inoculated in sterile 125 ml Erlenmeyer flasks with 75 ml of minimal medium plus 2% sucrose. The cultures were swirled in an Eberbach shaker at 120 rotations per minute at 30°C. Flasks were removed at intervals and the mycelia were filtered, washed with distilled water and dried in a stream of air. Extracts of these mycelia (table 5) showed increasing β-glucosidase from 40 to 96 hours in both activity per unit dry weight and in total activity. The growth media (filtrate) of these cultures also contained β-glucosidase activity which increased between 40 and 96 hours of growth. This increase roughly paralleled that of mycelia β-glucosidase but did not exceed one-tenth of the total mycelial activity of the cultures. It appears that mycelial β-glucosidase was primarily an endo-enzyme during this period of growth. Mycelia that has been gently filtered at 40 hours of growth did not lose detectable β-glucosidase activity with simple water washing. It is possible that the exogenous β-glucosidase in these cultures resulted from breakage of hyphae as the cultures were agitated. This point, however, is not established.

DISCUSSION

Relatively few studies have been made upon the exoenzymes of conidia and young mycelia. Zalokar and Cochrane ('56) showed that DPN-ase is primarily an exoenzyme in *Neurospora* conidia, and the production of this enzyme seems to be closely tied in with the process of conidial formation in this mold. Mandels ('51, '56) has shown that the invertase of *Myrothecium verrucaria* is released into the media as the conidia germinate and grow. Prior to liberation the invertase is

TABLE 5

Changes in β-glucosidase activity in growing Neurospora cultures[1]

Time of harvest[2]	β-Glucosidase: In mycelial extracts		In media
	100 mg mycelia	Total per culture	Total per culture
40	0.112	0.437	0.011
51	0.230	0.810	0.013
67	0.300	1.290	0.091
96	0.365	1.820	0.157

[1] Continuous method of assay: values are Δ O.D./min. at 410 mμ.

[2] Hours after inoculation.

located on the surface of these conidia. Inactivation experiments (Mandels, '53), indicated a surface location for invertase, maltase, trehalase, and cellobiase in *Aspergillus luchuensis* as well, although there were no attempts to extract these conidial enzymes. Jermyn ('53) also observed that β-glucosidase is liberated by *Stachybotrys atra* as the conidia start to germinate and grow.

The demonstration of β-glucosidase as an extra-cellular enzyme of *Neurospora* conidia is similar to Zalokar and Cochranes' ('56) findings with DPN-ase. The use of filtered dry conidia that were free of mycelia more clearly establishes the location of β-glucosidase at the surface of the conidia. It is not clear, as yet, whether the residual β-glucosidase released from conidia by acetone treatment is actually a different enzyme from that found in the first water extracts. Preliminary studies (unpublished) indicate, however, that there is no difference in the pH optima of these two β-glucosidase preparations. This residual activity may be analogous to the low levels of invertase released from bakers yeast by treatment with ethyl acetate (Myrback, '57).

The removal of β-glucosidase from conidia by distilled water probably parallels the release of the enzyme under natural conditions. This enzyme may have adaptive value in making β-glucosides nutritionally available to the mold, however, it is not clear if the natural substrates for the enzyme are derivatives of cellulose or of aryl-β-glucosides. The cellobiase, maltase and invertase activity in these conidial extracts may also have adaptive value for conidia growing in the presence of the corresponding disaccharides. In this connection the absence of cellulase from conidial preparations was not unexpected since Hirsh ('54) has shown that the cellulase of *Neurospora* is produced as an exoenzyme from mycelia grown at 35°C and not at 25°C, while the cultures described here were grown at 25°C.

The aryl β-glucosidase in *Neurospora* media did not exceed a tenth of the total activity of the culture during the first 4 days of growth. During this time liberation of the enzyme paralleled the synthesis aryl β-glucosidase within the mycelium. Similar observations (Jermyn, '55) have been made with *Stachybotrys atra* in which cellobiase activity was also predominantly in mycelia during the first 4 days of growth. This contrasts with the aryl β-glucosidase of *Stachybotrys atra* which was equally distributed between the mycelium and the media at 4 days of growth indicating the presence of two distinct β-glucosidases in this mold. In another case the release of aryl β-glucosidase by *Aspergillus luchuenses* (Reese and Levinson, '52) was detected after about 6 days of growth.

Studies with the invertase of yeast have suggested a possible origin for extracellular enzymes. Early experiments located invertase on the cell boundary of yeast (Dounce, '51; Myrback, '60), and later the location was narrowed to the cell wall or the space between the wall and the protoplast (Burger et al., '61). Both invertase and melibiase are synthesized within the protoplast then pass the cell membrane to the region of the cell wall (Friis and Ottolenghi, '59). There is a well defined difference in the ability of various yeast strains to excrete invertase (Wickerham, '58; Wickerham and Dorschack, '60). Unisexual strains were uniformly high in excretion (Wickerham and Dorschack, '60). An interesting result of these studies is the implied genetic control of enzyme excretion.

The recent success in protoplasts created experimentally from *Neurospora* conidia (Bachmann and Bonner, '59; Emerson and Emerson, '58) forms an interesting parallel with the yeast studies. These findings open the possibility that the excretion of enzymes can be studied in *Neurospora* and related fungi in a more direct and meaningful way.

SUMMARY

1. Conidia of *Neurospora crassa* were collected in a dry condition and washed with distilled water to extract exogenous enzymes.

2. An aryl β-glucosidase was highly active in conidial extracts while a relatively small residue of β-glucosidase in the conidia was released after treatment with cold acetone.

3. Alkaline phosphatase was found in water extracts of conidia, and much greater activity was released from conidia after acetone treatment.

4. Other enzyme activities extracted from the conidia were: maltase, invertase and cellobiase. Cellulase and lactase activities were not found in these extracts.

5. The distribution of β-glucosidase between mycelia and media was studied in growing cultures. Aryl β-glucoside remained mostly in the mycelia up to 94 hours of growth.

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The Cilioexcitatory Activity of Serotonin[1]

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Although a large number of substances are known to inhibit cilia, many of them irreversibly, the list of chemicals which promote or intensify ciliary movement is not imposing. Electrolytes have particularly complex actions. Both potassium and hydroxyl ions accelerate lateral gill cilia of lamellibranchiates (Gray, '28), but these effects are not recognized in non-molluscan mucous membrane. Magnesium ion inhibits *Mytilus* gill cilia (Gray, '28), but causes the peristomal cilia of *Stentor* to beat faster (Sleigh, '56). Veratrine and some of its component alkaloids stimulate gill cilia (Gray, '28; Aiello, '60). Although we have been unable to confirm the observation, topically applied ATP is reported to increase the activity of frog palatal cilia (Vorhaus and Deyrup, '53). The same membrane also appears to be excited by very low concentrations of penicillin (Blaich and Klar, '50). Among the chemicals which stimulate mammalian tracheal cilia when administered by mouth are some ingredients found in widely used expectorants, such as tribasic sodium citrate, sodium lauryl sulfate, and some of the Tweens (Dalhamn, '58). Local contact with negatively charged air ions promotes the movement of tracheal cilia (Krueger and Smith, '58). Although the observation has been disputed, epinephrine is reported to exert an excitatory action on some cilia (Blaich and Klar, '50; Kordik, Bülbring and Burn, '52; Ten Cate, Coomans and Walop, '55). None of these diverse effects, however, has been as widely studied as the cilio-acceleration produced by acetylcholine and its congeners (Plattner and Hou, '31; Blaich and Klar, '50; Kordik et al., '52; Bülbring et al., '53).

Probably some of the reported effects of drugs on ciliary activity are spurious, particularly in the case of cilioaccelerator substances. Many ciliated membranes appear to be stimulated by even the gentlest attempts to wash off the overriding film of mucus. The mechanism of this mechanical excitation has been clarified in the case of the frog palate (Lucas, '35), but tactile and other mechanical stimuli sometimes elicit increased activity in preparations where cilioaccelerator nerves are not recognized (Umeda, '29). It is practically impossible to apply chemicals to isolated ciliated tissues without changing at least transiently physical factors in the environment. Also unevaluated as a source of misinterpretation is the possibility that some drugs act only indirectly through changes in the rate of mucus production or in the consistency of the mucus sheet. This consideration is especially pertinent whenever methods of particle transport are employed as the only measure of ciliary activity. There is no simple solution to this dilemma because mucus-secreting cells are found in intimate association with ciliated cells in all or almost all metazoa. For these reasons the conclusions in this report are based on determinations of ciliary beat frequency. Since wide variations in the viscosity of the medium do not influence beat frequency (Gosselin, unpublished data), possible changes in the quantity and quality of mucus are believed to be irrelevant in this study.

Of all cilioaccelerator substances described in the literature, we have found none to be so potent as serotonin (5-hydroxytryptamine), as tested on the cilia of lamellibranch mollusks. Since our original observations (as first reported to the American Society for Pharmacology and

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Experimental Therapeutics in the fall of 1958), we have discovered that Aiello ('57) was also aware of this stimulating effect of serotonin on *Mytilus* gill cilia. Subsequently Tsuchiya and Kensler ('59) reported that the same indole amine excites mammalian cilia as represented by enhanced particle transport on excised rabbit tracheal mucosa.

Our own observations have now led us to believe that at least in lamellibranch gills the physiological control of ciliary activity may be based on a serotonergic mechanism. The concept of a "local hormone," exerting its actions in the same tissue which synthesizes and destroys it, has been elaborated by Burn ('56) and applied to the problem of autorhythmicity in the heart and in ciliated epithelium. For reasons which will be published elsewhere, we believe that serotonin is a better candidate for this role than the acetylcholine proposed by Kordik et al. ('52). In establishing this hypothesis it is necessary first to define precisely the cilioaccelerator activity of serotonin, as is done in this report for the gill cilia of various lamellibranch mollusks.

MATERIALS AND METHODS

Specimens of *Modiolus demissus* were shipped from the Marine Biological Laboratories at Woods Hole. *Mytilus edulis* was collected at Ipswich, Massachusetts. *Anodonta spp.* was obtained from various fresh water lakes and ponds near Hanover; specimens were kept in the laboratory in a stream of running tap water. Both species of marine mussels were stored in the laboratory in a moist container at 4°C. Refrigerated specimens remained viable for at least one week. *Modiolus* proved to be especially hardy; it was often usable even after three weeks of storage.

The entire gill was excised and immersed in an appropriate salt solution. Artificial sea water at pH 7.8 (Marine Biological Laboratory formula no. 4) was used for marine species. Because pond water proved to be destructive to excised *Anodonta* gill, a fresh-water molluscan Ringer's solution was devised by using chloride salts of the following cations in accordance with the analysis of Hayes and Pelluet ('47): sodium 15.47 mM, potassium 0.40 mM, calcium 5.29 mM, and magnesium 0.35 mM (final pH 6.9). In salt solutions at room temperature, freshly cut molluscan gills secrete large quantities of mucus. Single lamellae teased from the excised gill were left in sea water or Ringer's solution for at least half an hour to enable them to shed this mucus. Thereafter the gill filaments could be handled gently without stimulating excessive mucus production.

The method of mounting gill fragments for microscopic visualization depended upon which cilia were chosen for study. Observations reported here were confined to the lateral cilia. A portion of a single plate (lamella) consisting of less than a dozen parallel filaments was transferred to a drop of sea water or Ringer's solution on a large rectangular coverslip (22 × 40 mm, no. 2 thickness), so that each filament rested on its abfrontal surface. Over the preparation was laid a small square of woven Nylon mesh (widely sold as curtain material) with a hole cut in the middle of such a size as to accommodate the gill filaments. This mesh served to fence in the specimen so that it could not crawl away or be washed away; it also acted as a shim for a square coverslip (22 × 22 mm) which was placed on top. White petrolatum was applied to the two outer edges of the top coverslip to seal it to the bottom coverslip. Through one of the unsealed edges fresh sea water could be drawn by the capillarity of a piece of filter paper held against the opposite edge. In this way the tissue was superfused at 5-minute intervals with about 1 ml of fresh sea water, in which serotonin was dissolved when appropriate. This superfusion was accomplished without moving the tissue or interrupting microscopic observation. In terms of the stability of the beat frequency, intermittent superfusion was found to be superior to continuous superfusion.

One or more assembled preparations were mounted on top of a transparent cell which rested directly on the microscope stage. The temperature of the cell was controlled by water pumped through it. In most experiments the temperature was held at 20°C, but occasionally temperatures as high as 25° and as low as 18° were employed.

Technical details relating to the stroboscopic method of measuring the beat frequency of lateral cilia will be described elsewhere. In summary the preparation was illuminated through a substage condensor of long focal length by a mercury vapor lamp mounted in the rear focal plane of the condensor. The flash frequency of the lamp was controlled by a calibrated electronic circuit (Grass Photo-Stimulator S-1). The flash duration was constant at 10 microseconds, while the frequency was continuously variable between one and 100 flashes per second. In adjusting the stroboscope until metachronal waves of the lateral cilia appeared to standstill, care was taken to avoid harmonics and subharmonics of the biological rhythm. Of the various cilia studied in this laboratory only lateral cilia of lamellibranch gills have proved suitable for the accurate determination of beat frequency by stroboscopic methods. The movements of other cilia were too slow, too irregular, or too uncoordinated. In most preparations of lateral cilia the uncertainty of a single frequency measurement was less than ± 0.5 cycles per second (cps). Because beat frequencies differed in an apparently random fashion along adjacent segments of even a single lateral track, it was necessary to restrict observations to carefully defined target areas of small size. Regions of stablest beat frequency were found on those filaments near the outside edge of the mounted gill plate, probably because the chemical environment of the cells was most constant there. In each preparation several areas were selected for study. After 30 to 60 minutes of intermittent superfusion, the ciliary motion in most target regions had settled down to a comparatively low and constant frequency.

In a few experiments the mean metachronal wavelength of lateral cilia was determined repeatedly. Each measurement was made on the projected image of a single-flash microphotograph taken through a trinocular head on fast 35-mm film (Kodak Tri-X, Agfa Isopan Record). Recorded values represent the distance in microns along the lateral track occupied by the average metachronal wave in several dozen successive waves.

In all cases 5-hydroxytryptamine was used as its creatinine sulfate salt (obtained from Abbott Laboratories). Drug solutions and the balanced salt solutions from which they were prepared did not differ in pH.

RESULTS

Figure 1 depicts responses of the lateral cilia from 4 separate regions of an excised and isolated gill plate (ctenidium) of *Mytilus edulis* when serotonin creatinine sulfate (5-HT) was added to the artificial sea water (pH 7.8) bathing the tissue. In each case exposure to 5-HT caused a

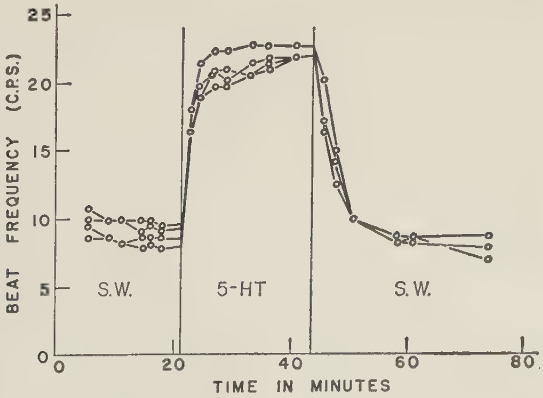

Fig. 1 Beat frequency of lateral cilia in 4 separate regions of an excised gill plate of *Mytilus edulis* during exposure to sea water (S.W.) and to sea water containing 5-HT (10^{-6} gm/ml).

prompt rise in beat frequency, which soon stabilized at a level much above the control. Even in experiments where the superfusion was brisk and continuous instead of intermittent, more than a minute elapsed before the beat frequency reached its new plateau. Thereafter the accelerated rate was sustained as long as the concentration of 5-HT was maintained. In every case the response was completely and promptly reversible.

By exposing preparations to varying concentrations of 5-hydroxytryptamine, dose-response curves were constructed, as seen in figure 2. These data, which were obtained in the later spring and early summer of 1958, illustrate the variability in responsiveness of preparations of *Mytilus edulis*. Each point represents the sustained response of one target area or the average response of several target areas within the same preparation. Each series of points describes an S-shaped curve. The threshold concentration is not well defined, but at least some preparations accelerated in response to concentrations as low as 5 × 10^{-10} M. The average ED_{50} lay near 10^{-8} M. The maximal response, usually obtained with concentrations of about 10^{-7} M, was no greater than tha encountered occasionally in gills exposed to no 5-HT or other known cilioaccelerator substances. Preparations with a high auto rhythmicity responded to added serotonin with smaller increments in beat frequency than seen in figure 2, but in terms of the threshold concentration they appeared to be no less sensitive, as for example in figure 3.

Figure 3 also illustrates the inhibitory action of high concentrations of 5-HT on summer *Mytilus*. The beat frequency of lateral cilia declined during exposures to concentrations of 2.5 × 10^{-6} M (10^{-6} gm/ ml) or above. Unlike the cilioaccelerator activity of low doses, this inhibition developed gradually only after an appreciable latency; similarly recovery in sea water was slow but eventually complete. After such an intense exposure gill preparations were found to be relatively insensitive to concentrations of 5-HT which beforehand had proved adequate to produce distinct stimulation. The mechanism of this refractoriness has not been investigated.

Figure 3 also records measurements of the mean metachronal wavelength dis-

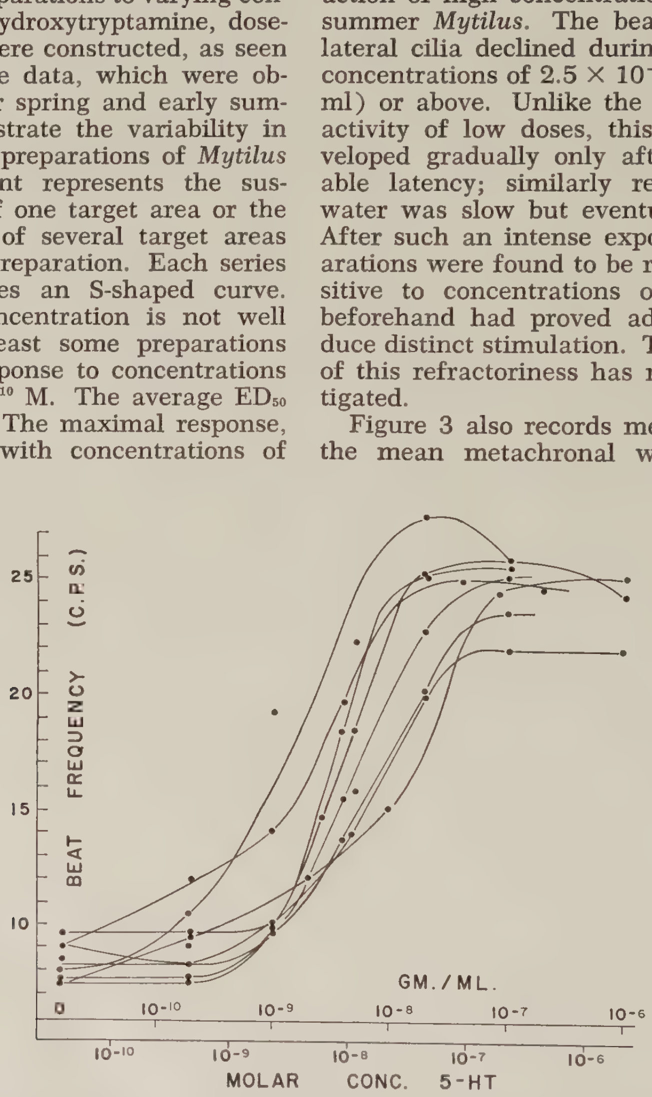

Fig. 2 Influence of 5-HT concentration on the beat frequency of lateral cilia on excised gills of *Mytilus edulis*. Each curve represents a different preparation. Only summer specimens are included.

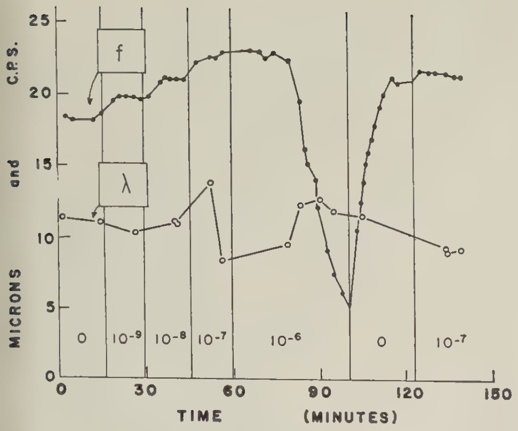

Fig. 3 The beat frequency (f) and metachronal wavelength (λ) of lateral cilia on the excised gill of a summer specimen of *Mytilus* during exposure to various concentrations of 5-HT (in gm/ml of sea water).

played by these lateral cilia as their beat frequency was both raised and depressed by 5-HT. Although this parameter was less constant here than in most experiments, the changes encountered were erratic and evidently unrelated to shifts in beat frequency, an impression which was supported by other trials. According to the wave equation, therefore, the effect of 5-HT on the velocity of metachronal wave transmission was in essentially every instance proportional to the change in beat frequency.

The lateral gill cilia of *Modiolus demissus* were also stimulated by the presence of 5-HT added to the surrounding sea water. The dose-response curve (fig. 4) is similar to that seen with *Mytilus*, but *Modiolus* proved to be less sensitive in that it required higher drug concentrations; the ED_{50} averaged about 3×10^{-7} M. Another marked difference was the failure of high concentrations of 5-HT to inhibit *Modiolus* lateral cilia. Consequently each preparation exhibited a long response plateau, spanning at least a hundred-fold range in the drug concentration. This maximal frequency differed from one preparation to the next and from one portion of any gill to an adjacent portion in an apparently random fashion. No frequency gradients could be detected along the filaments.

Differences between *Mytilus* and *Modiolus* were less conspicuous when these mussels were collected and studied in the winter. In terms of its response to 5-HT, the winter *Modiolus* did not differ signifi-

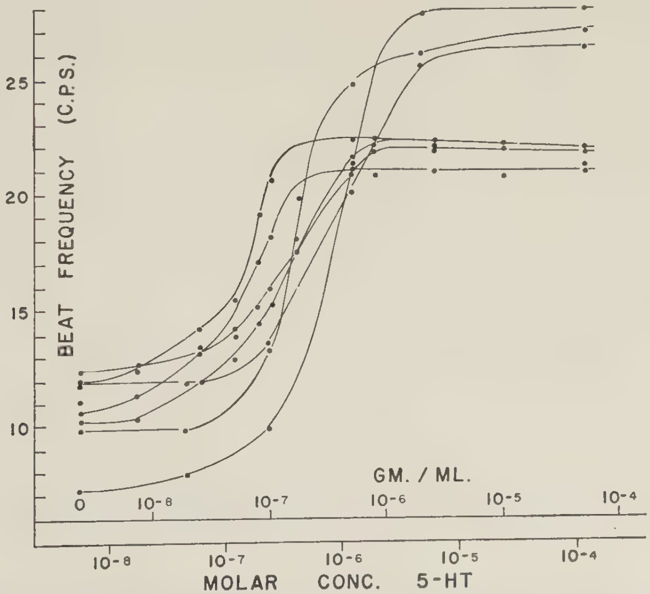

Fig. 4 Influence of 5-HT concentration on the beat frequency of lateral cilia on excised gills of *Modiolus demissus*. Each curve represents a different preparation.

cantly from the summer specimens shown in figure 4. *Mytilus*, however, lost in the wintertime its high sensitivity to the cilioacceleratory action of 5-HT (cf. figs. 2 and 5) and approached the sensitivity characteristic of *Modiolus*. At the same time ciliary inhibition encountered with high drug concentrations was much less marked in winter *Mytilus* than in summer specimens, even when the mussels were stored in the same way and tested at the same laboratory temperature. Consequently dose-response plateaus were attainable (fig. 5), and maximal frequencies were sustained almost indefinitely in the presence of 5-HT concentrations which caused progressive inhibition in the summertime.

A fresh-water species of the genus *Anodonta* proved to be less sensitive to 5-HT than the two marine mussels, but with high drug concentrations intense stimulation was observed (fig. 6). Like *Mytilus*, the lateral cilia of the summer *Anodonta* gill were inhibited gradually by high concentrations of this indole amine, so that no sustained maximal frequency could be defined. Seasonal variations in the responsiveness of *Anodonta* were no investigated because winter specimens were unavailable.

DISCUSSION

The data reported here concern solely the serotonin response of lateral cilia present on the lamellibranch gill. It has become apparent, however, that other molluscan gill cilia are also stimulated by serotonin (5-hydroxytryptamine, 5-HT). Experiments on particle transport (Gosselin and O'Hara, '61) reveal the cilioacceleratory action of 5-HT on frontal cilia and a comparable effect on the giant abfrontal cilia of *Mytilus* and *Modiolus* has been detected on high-speed movie film (Gosselin and Campbell, study in progress). Although a positive response by the frontolateral cilia has not been established, 5-HT appears to exert a general stimulatory action on the gill cilia of both marine and fresh-water lamellibranchiates.

The action of 5-HT on non-molluscan cilia, however, is still controversial. Our own studies on frog palatal cilia, both

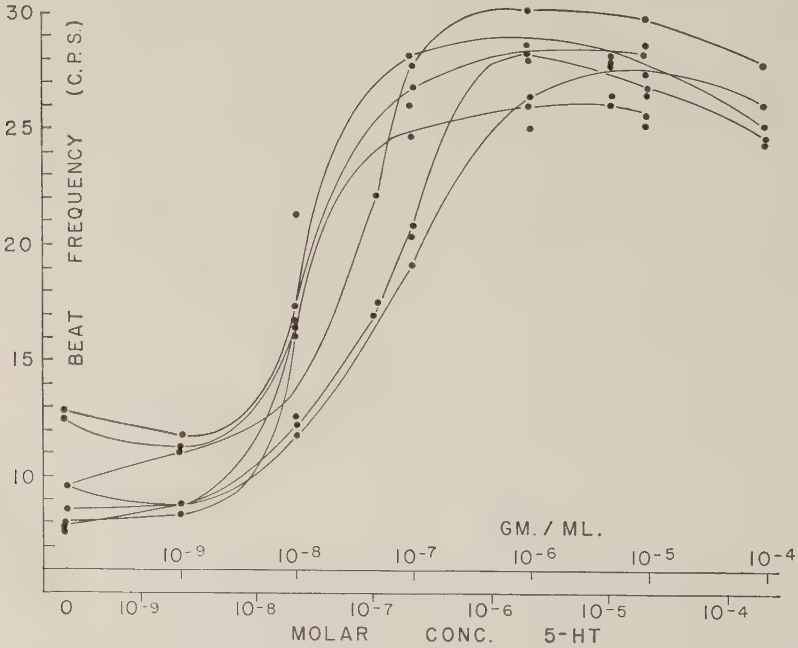

Fig. 5 Influence of 5-HT concentration on the beat frequency of lateral cilia on excised gills of *Mytilus edulis*. Each curve represents a different preparation. Only winter and spring specimens are included.

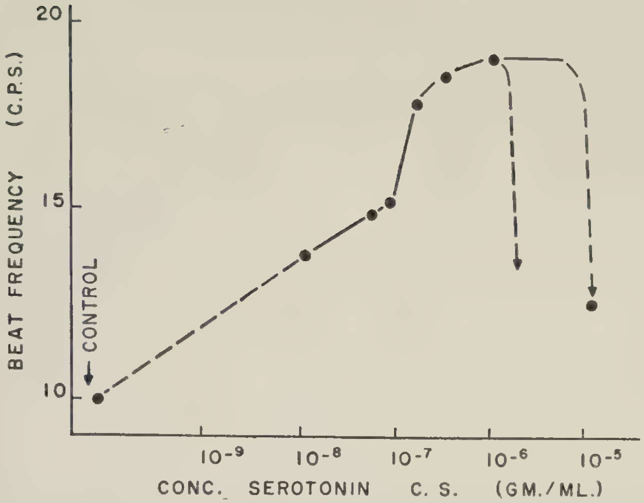

Fig. 6 Beat frequency of the lateral cilia on a single specimen of excised *Anodonta* gill at various concentrations of 5-HT, demonstrating the instability of the response at drug concentrations of 10^{-6} gm/ml and higher.

in situ and in excised strips, indicate that 5-HT in concentrations as high as 5 × 10^{-5} M exerts no consistent action on the velocity of particle transport across this mucous membrane. With the excised rabbit trachea, Tsuchiya and Kensler ('59) report a 20% increase in the velocity of particle transport when the preparation is bathed in 10^{-9} M 5-HT in Tyrode's solution at 38°C. On the other hand Kimura ('59) observed no gross influence on the cilia of rat tracheal rings during a 10-minute exposure to 1% 5-HT. Finally Kreuger and Smith ('58, '60), measuring the beat frequency of rabbit and guinea pig tracheal cilia *in situ*, have concluded that 5-HT inhibits cilia and that the mobilization of endogenous 5-HT may be responsible for the cilio-inhibition which they observed when the tracheal mucosa was exposed to air containing positively-charged ions. Even if their inferences implicating serotonin are valid, the observed cilio-deceleration may have arisen through very complex and indirect mechanisms, such as general vasoconstriction and tracheal ischemia. Our own studies on the serotonin response of mammalian cilia are not sufficiently advanced to resolve the issue.

Beat frequency as reported here in gill cilia is believed to be one of the least ambiguous measures of ciliary movement, but it is of course only one aspect of the problem. The amplitude and force of beat are also important parameters, but they cannot be measured with an acceptable degree of accuracy in most preparations. Although we have been able to recognize in photomicrographs of lateral gill cilia gross reductions in the amplitude of beat produced by raising the viscosity of the bathing fluid (Gosselin, '58), lateral cilia in general do not lend themselves to satisfactory estimations of beat amplitude. In our experience the same is true of frontal gill cilia, frog palatal cilia, and mammalian tracheal cilia. The large abfrontal cilium of *Modiolus* responds to the presence of 5-HT by increasing its beat frequency without changing the amplitude of beat (unpublished data).

Other measurable parameters of ciliary movement are related to the metachronal pattern created by the sequential beating of neighboring cilia. As noted in figure 3 and in the text, 5-HT does not modify in any consistent or regular way the mean metachronal wavelength of lateral cilia on *Mytilus* or *Modiolus* gill, in agreement with the conclusion of Aiello ('60). Accordingly elevations of beat frequency observed with 5-HT reflect proportional rises in the velocity of metachronal wave transmission. Although small changes in metachronal wavelength have been reported in protozoa exposed to various drugs and electrolytes (Sleigh, '56), we have failed to find any substance which produces by a chemical action on the lateral cilia of the lamellibranch gill a significant dissociation of wave frequency and wavelength.

Because the reaction of lateral cilia to 5-HT is so prompt, sustained, and reversible, it might be employed as the basis of a bioassay for serotonin. Of course the inherently shallow slope of the response-dose curve (fig. 2) is a serious and unalterable handicap, but perhaps only in this respect does the procedure compare unfavorably with the rat fundus preparation of Vane ('57). With an excised piece of gill as the test system less than 1 ml of solution is required for the analysis. In terms of its inherent sensitivity *Mytilus* gill is preferable to *Modiolus*, but because exposures to supraoptimal concentrations of 5-HT inhibit the cilia of summer *Mytilus* (fig. 3) and reduce their subsequent sensitivity to low concentrations of this amine, *Modiolus* gill is probably more satisfactory as a test system. Neither the kinetics nor the mechanics of the refractory state in *Mytilus* has been established, but specimens collected in the winter and spring exhibit neither the inhibition nor the post-exposure lack of responsiveness. Perhaps relevant to these phenomena are the well established seasonal variations in the water and glycogen contents of molluscan gill (Usuki and Koizumi, '54).

Maximal beat frequencies found in gills exposed to optimal concentrations of 5-HT were no higher than the rates encountered occasionally in control gills. Thus cilia proved to be incapable of generating a faster rhythm under the influence of 5-HT than under the control of unknown endogenous stimuli. That the normal physiological stimulus may be endogenous serotonin is an attractive hypothesis in support of which much evidence can be marshalled. As will be reported elsewhere, gill plates of *Mytilus* and *Modiolus* not only contain extractable 5-HT but also possess enzymes for its production and degradation. Exogenous 5-HT stimulates both the aerobic and anaerobic metabolism of the gill and promotes glycogen breakdown. Presumably these metabolic effects are related to the cilioacceleration demonstrated here.

Although an action of 5-HT on vertebrate cilia has not been established, this indole amine has been shown to stimulate the autorhythmicity of some other contractile tissues in the mammal. Wooley and Shaw ('57) regard the ability of serotonin to accelerate the rhythmic pulsations of glial cells in tissue culture as a possible reflection of its physiological role in the brain. Ludány et al. ('59) report that 5-HT increases the frequency of spontaneous rhythmic motion in the intestinal villi of anesthetized dogs. Its actions on the mammalian heart are complex and as yet imperfectly understood, but it usually increases the rate of isolated hearts (Trendelenburg, '60). Apparently serotonin exerts positive chronotropic effects on a wide variety of autorhythmic contractile mechanisms.

SUMMARY

Serotonin (5-hydroxtryptamine, 5-HT) in low concentrations is able to increase the beat frequency of lateral gill cilia of *Mytilus edulis* and of other lamellibranchiates. The acceleration, as measured in stroboscopic light, was prompt, sustained and graded over a wide range of drug concentrations. Maximal frequencies, however, were no greater than sometimes encountered in untreated gills (25–35/sec.). Dose-response curves were established. Preparations varied widely in sensitivity but summer *Mytilus* usually responded to lower concentrations (about 10^{-9} M) than *Modiolus* or *Anodonta*, and a maximal response required only 10^{-7} M. Higher concentrations inhibited the ciliary activity of summer *Mytilus* but not the lateral cilia of the other species studied. Unlike the stimulatory response to low concentrations of 5-HT, this inhibitory reaction developed only after a considerable latency and persisted for a long time after removing the supply of exogenous 5-HT. In the winter *Mytilus* was less sensitive than in the summer to the cilioacceleratory action of 5-HT and an inhibitory response could not be elicited. No consistent changes in metachronal wavelength accompanied these 5-HT-induced changes in beat frequency. These results may reflect a physiologica

role of endogenous 5-HT in controlling the activity of molluscan gill cilia.

ACKNOWLEDGMENTS

The author is grateful for the technical assistance of Miss Matilda Ernst and Miss Gail O'Hara in many phases of this study. To Miss Ann Campbell is due credit for the experiments on the frog palate. Dr. A. O. Geiszler of Abbott Laboratories generously furnished the supply of serotonin creatinine sulfate. Finally it is proper to recognize the author's indebtedness to Dr. R. R. Ronkin of the University of Delaware, who first demonstrated to him the elegance of the lamellibranch gill as an object for the study of ciliary mechanisms.

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Inhibition and Recovery of Golgi Response in Intestinal Epithelial Cells of the Rat Injected with Monoiodoacetic Acid-Treated Fat-Induced Chyme[1]

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Transfer of chyme from the gut of a fat-fed donor rat to the intestine of a starved recipient animal elicits a striking increase in prominence of the Golgi apparatus of the intestinal epithelial cells of the recipient (Adamstone and Taylor, '59). By contrast, this response fails to occur in recipient animals which have been injected intraperitoneally with an adequate dose of monoiodoacetic acid (MIA) (Taylor and Adamstone, '60). However, the injection of MIA also produces profound physiological disturbances sometimes terminating in death. Hence, the present experiments have been carried out to determine whether much smaller doses of MIA added directly to the transferred chyme could produce the same inhibitory effect on the Golgi apparatus without such serious reactions in the recipient. In addition, a second series of experiments has been carried out to determine whether the use of MIA produced permanent damage in the epithelial cells or whether recovery could occur.

I. *Experiments involving transfer of MIA-treated chyme*

PROCEDURE

Chyme for transfer was secured from the jejunum of donor rats fed 0.25 cm^3 olive oil colored red with Sudan IV 25 minutes before sacrifice. From each chyme sample two lots were prepared for transfer each containing 0.1 cm^3. One of these was transferred untreated and the other was mixed before transfer with 0.01 cm^3 aqueous solution of MIA of known strength so that the amounts actually added to the chyme sample varied between 0.0001 and 1.0 mg (table 1).

The recipient animals were starved for 24 hours and the chyme samples were transferred under nembutal anesthesia to short ligated segments of the jejunum using methods similar to those previously described (Adamstone and Taylor, '59). In a few cases, chyme was obtained some time before use and was kept frozen until needed (table 1, animals 7 and 9). Tissue samples were secured for study from the intestine of donor and recipient, as follows: (1) material from the donor, at autopsy, (2) blebs of tissue from the ligated jejunal segments of the recipient before chyme injection, (3) tissues from the injected segments of jejunum which had received the treated and untreated chyme, (4) control material from an adjacent empty region of the gut of the recipient at termination of the experiment. In each case one tissue sample was placed in Ringer's solution for examination of fresh cells and another was fixed in Lavdowsky's F.A.A., (Formol-acetic-alcohol) for the later preparation of hematoxylin-stained sections. No material was prepared for silver impregnation since our earlier studies have shown that the negative image of the Golgi apparatus as seen in hematoxylin-stained preparations gives a truer picture of the Golgi response of intestinal epithelial cells to fat absorption than the classical silver impregnation technique.

For microscopic examination, the fresh epithelial cells were teased apart in Ringer's solution on a microscope slide and the mount sealed with a vaseline-rimmed coverslip. In each sample the percentage

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[2] With the technical assistance of Ralph A. Jersild.

of positive cells (showing a prominent Golgi apparatus) was determined by making a count of the first 100 clearly visible cells. The fixed material was embedded in paraffin and 10-μ sections, cut longitudinally, were prepared and stained with Heidenhain's iron alum hematoxylin for study of the negative image of the Golgi apparatus.

EXPERIMENTAL OBSERVATIONS

The findings from microscopic examination of fresh preparations showed that the Golgi apparatus of cells from donor animals gave the usual positive response (fig. 1). In recipient animals, control material taken from the gut both before the injection of chyme (fig. 2), and at the end of the experiment (fig. 4), showed a highly negative picture. By contrast, cells from the ligated sections of the gut containing untreated chyme were highly positive (fig. 5), but, in the segment containing MIA-treated chyme, there was a wide variation in the proportion of positive cells ranging from an almost completely positive response in animals which had received the lowest dosage of MIA, to a preponderantly negative response in animals which had received 0.2 to 0.1 mg MIA in 0.1 cm^3 chyme (fig. 3). See table 1.

Prepared sections of this material stained with iron alum hematoxylin paralleled these findings very closely, i.e., a prominent negative image of the Golgi apparatus was to be seen in cells from donor tissues (fig. 6), and also from the segment of the gut of the recipient containing untreated chyme (fig. 10), but the recipient control material (figs. 7 and 9) as well as cells from the gut segment containing chyme treated with an adequate amount of MIA (fig. 8), showed little or no indication of the negative image of the Golgi apparatus. However, in cells from the injected areas in which an insufficient dose of MIA had been present, a negative image was clearly evident in many cells.

Effective concentration of MIA. From the data presented in table 1, as well as from study of stained sections, it is evident that when at least 0.2 mg MIA was added to 0.1 cm^3 chyme secured from a fat-fed donor animal, a positive response of the Golgi apparatus was not observed. This quantity of MIA may, therefore, be regarded as near the threshold dosage and it is very small in contrast to the amount used in our previous studies (Taylor and Adamstone, '60) as well as by Verzar ('36) and Frazer ('48) but it proved to be equally effective in preventing the Golgi response to injected chyme. However, in the recovery tests to be described below, double this concentration of MIA has been used to ensure inhibition of the Golgi response and the effectiveness of this dosage

TABLE 1

Response of the Golgi apparatus of epithelial cells from isolated segments of the intestine of the rat to untreated chyme from a fat-fed donor and to chyme treated with MIA

Exp. no.	Amount MIA mg/0.1 cm^3 chyme	Per cent positive response				
		Donor	Recipient			
			Control before	Treated chyme	Untreated chyme	Control after
1	0.0001	96	3	98	97	3
2	0.01	98	4	59	91	5
3	0.01	98	3	73	78	6
4	0.01	95	4	68	73	4
5	0.01	97	4	76	98	33
6	0.01	97	3	31	50	4
7	0.1	—	3	20	—	2
8	0.1	97	3	65	87	7
9	0.15	—	3	23	20	6
10	0.2	—	5	3	97	5
11	0.2	97	2	1	—	0
12	0.2	—	8	12	91	10
13	0.2	99	0	7	80	2
14	1.0	97	1	3	90	0

has also been checked by experiment. This was considered necessary since it is difficult to be sure of the length of the ligated segment of the gut because of variations in degree of contraction and tonus of the gut muscle.

II. Experiments on recovery of cells from MIA poisoning

PROCEDURE

In this series of experiments, poisoning of the intestinal epithelial cells was effected by injecting an aqueous solution of MIA (0.4 mg per 0.1 cm^3 distilled water) into a ligated segment of the jejunum using just enough of the solution to fill, but not distend, the segment of the gut. This was done by operation under nembutal anesthesia and the MIA was allowed to remain in the gut for 30 minutes. In the animals of group A, the MIA was removed after loosening the ligatures by washing out the injected and neighboring portions of the gut with warmed saline solution. In the remaining animals of group B, the injected MIA solution was greatly diluted by injecting an additional 0.5 cm^3 warm saline, after which as much fluid as possible was withdrawn from the ligated segment using a hypodermic syringe. The loosened ligatures were left in place to mark the treated area.

Following removal of the MIA in group A, 0.1 cm^3 fat-induced chyme was injected immediately into a ligated portion of the MIA-treated area and into an adjacent, untreated, but washed-out segment which served as a control. In group B after removal of the MIA, the abdominal incision was closed with sutures and the animal was allowed to recover for a period of zero to 4 hours. At the end of this recovery period the animals were fed 0.2 cm^3 olive oil colored with Sudan IV and after a further interval of 30 to 90 minutes, which was allowed for the oil to pass down the gut into the treated area, the experiment was terminated.

At autopsy, tissue samples from the MIA-injected, and untreated control areas of the gut were removed for examination in the fresh condition and for fixation in Lavdowsky's F.A.A. for later preparation of hematoxylin stained sections. Again the fresh cells were teased out in Ringer's solution and a count was made to determine the percentage of cells showing a positive Golgi response.

EXPERIMENTAL OBSERVATIONS

In the animals of group A, in which the experimental segment of the gut had been washed out before chyme injection, there was a wide variation in the Golgi response of the fresh cells to the injection of chyme. In the material from the control area, the positive count ranged from 24 to 93% and in the MIA-treated segment from 33 to 74%. In the animals of group B which were fed olive oil following a more or less prolonged recovery period after removal of the MIA, the response ranged from 42 to 100% in the control areas and from 37 to 100% in the MIA-treated experimental material (figs. 11 and 12). These results are summarized in table 2. Two animals of this group, however, (B6 and B7) failed to show a positive response indicative of recovery and in the sectioned material there was definite evidence of cell injury and sloughing at the tips of the villi in animal 7.

In hematoxylin-stained sections of this material the prominence of the negative image of the Golgi apparatus varied greatly but in general it paralleled the prominence of the positive image in the fresh cells being most striking and abundant in animals showing the largest percentage of positive fresh cells and least conspicuous in animals showing a low percentage of positive fresh cells.

The findings in both of these experiments indicate that there is definite evidence of recovery of the intestinal epithelial cells from the effects of MIA poisoning in most cases, as indicated by the ability of the Golgi apparatus to respond to the ingestion of fat or fat-induced chyme. However, in the two animals which failed to respond, removal of the MIA may not have been sufficiently thorough or the rigors of the operative technique may have been too injurious to these animals. In spite of these two adverse results, it seems evident that, given sufficient time, recovery is possible in most cases, and it is noteworthy that in animals B8 and B10 in which recovery was apparently complete, the Golgi image in fresh cells and the negative image

TABLE 2

Recovery of Golgi response to fat ingestion in intestinal epithelial cells of the rat after treatment with MIA[1] *and its subsequent removal*

Exp. no.	Time MIA in gut	Recovery period after MIA removal	Food	Time injection or feeding to sacrifice	Positive Golgi response: Control area	Positive Golgi response: MIA-treated area
	min.	*min.*		*min.*	%	%
A1	30	0	chyme	30	24	43
A2	30	0	chyme	30	93	33
A3	30	0	chyme	30	78	74
A4	30	0	chyme	30	52	37
B5	30	0	olive oil	30	75	37
B6	30	30	olive oil	30	3	5
B7	30	60	olive-oil	60	0	0
B8	30	140	olive oil	50	100	100
B9	30	210	olive oil	60	89	73
B10	30	220	olive oil	50	99	93
B11	30	230	olive oil	90	60	49
B12	30	230	olive oil	90	42	48

[1] A 0.4 solution of MIA (twice minimal effective dose) was injected into the gut of each animal at the beginning of the experiment.

in hematoxylin-stained sections were as striking and prominent as usually seen in experiments with normal untreated animals.

DISCUSSION

The first group of experiments were carried out to determine whether minimal amounts of MIA added to fat-induced chyme from donor rats could block the Golgi response in absorptive intestinal epithelial cells after injection into the gut of recipient animals and at the same time avoid the severe disturbances which accompany intraperitoneal injection of MIA. These experiments have shown that the epithelial cells are very sensitive to very small doses of MIA ranging down to 0.2 mg MIA per 0.1 cm^3 chyme, i.e., approximately 0.2%. The effect, in so far as inhibition of the Golgi response is concerned, is quite comparable to that produced by a much larger dose administered intraperitoneally. For example, rats weighing 150 to 250 grams would require 12 to 20 mg MIA if the drug were administered at the level of 0.08 mg per gram body weight, as suggested by Verzar ('36). This is 60 to 100 times the amount which, when added to injected chyme, will produce inhibition of the Golgi response in a limited area of the gut. As a result, the generalized toxic effects to which Frazer ('48) objected, can be completely eliminated.

In the second group of experiments, there is considerable variation in the degree of recovery from the effects of MIA poisoning, but the results appear to justify the conclusion that in most cases the cells do not suffer permanent damage. Ohnell et al. ('39), on the other hand, found that the use of MIA in larger doses was followed by extensive injury to the intestinal epithelial cells.

In the experiments in which a Golgi response occurred there was considerable variation in the percentage of positive cells. Several factors may be responsible for this, including the severity of the operative technique and the washing of the gut, variations in contact of the epithelial cells with the chyme due to differences in motility and distention of the gut, and finally, a lowered stimulating effect of the chyme due to depletion of fat content by the donor, Under the circumstances, variation in the Golgi response is not surprising, but it is still uncertain as to whether MIA produces a direct inhibition of the Golgi response, or, whether failure of the Golgi response follows inhibition of fat absorption.

SUMMARY AND CONCLUSIONS

The present experiments, which involve the administration of small quantities of monoiodoacetic acid to recipient rats by injecting the substance into a small ligated segment of the gut after mixing it with chyme from fat-fed donor animals, have shown that:

1. A minute quantity of this substance (MIA), viz., 0.2 mg in 0.1 cm^3 chyme, is sufficient to inhibit absorption of fat and the usual marked increase in prominence of the Golgi apparatus.
2. The inhibitory effect of MIA administered in this manner is restricted to the segment of the gut into which it has been injected and does not affect neighboring areas injected with untreated chyme.
3. The addition of minimal doses of MIA to the food appears to eliminate completely the general toxic effects of the drug while its inhibitory effects are not impaired.
4. Removal of MIA from treated segments of the gut is usually followed by more or less complete recovery and a normal response of the Golgi apparatus.

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PLATE 1

EXPLANATION OF FIGURES

1 Prominent Golgi apparatus in fresh intestinal epithelial cells from donor animal fed olive oil 30 minutes before sacrifice.

2 Fresh cells from control bleb of intestine of recipient before injection of MIA-treated chyme. The Golgi apparatus is not evident.

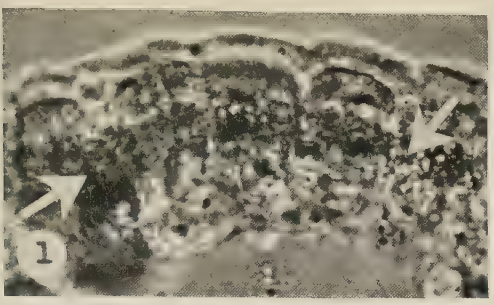
3 Failure of Golgi response in cells from intestine of recipient 25 minutes after injection of MIA-treated chyme. Compare figure 5.

4 Fresh cells from uninjected control area of intestine of recipient adjacent to segment which received MIA-treated chyme. No Golgi apparatus can be seen.

5 Prominent Golgi response in fresh epithelial cells from segment of intestines injected with untreated chyme. Compare figure 3.

6–10 Hematoxylin-stained sections of epithelium from portions of gut illustrated in figures 1–5. Note that a clear-cut negative image of the Golgi apparatus can be seen only in material from the donor and from the recipient of untreated chyme.

11, 12 After recovery period, fat-fed rat showed a prominent Golgi apparatus in cells from untreated area of the gut (fig. 11), and also in cells from the area originally treated with MIA and into which fat had descended (fig. 12).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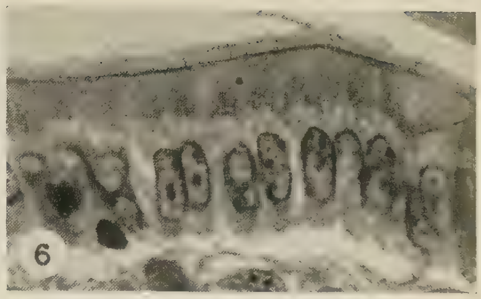
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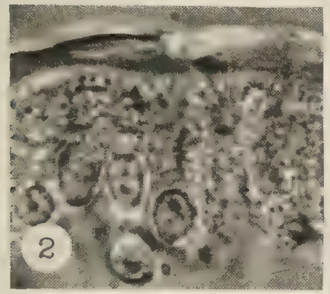
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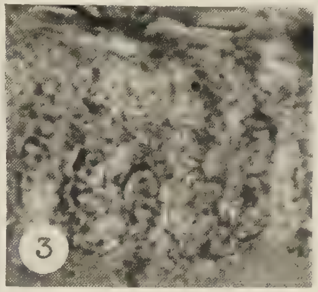
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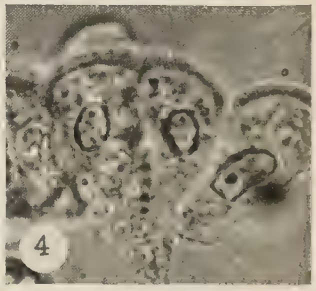
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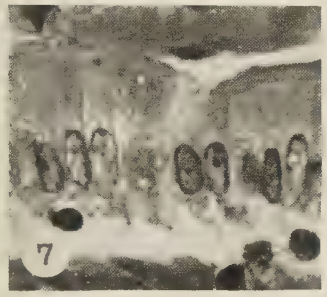
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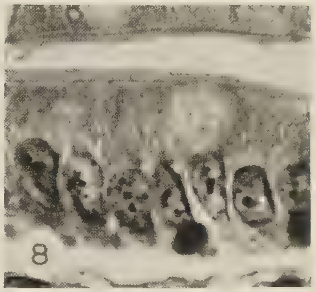
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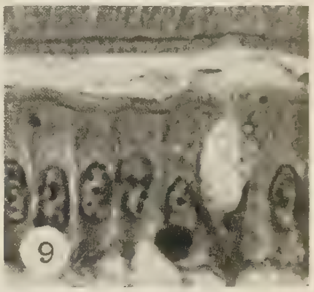
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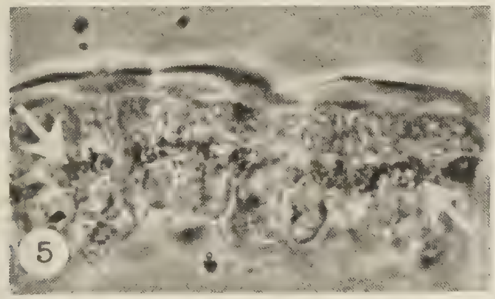
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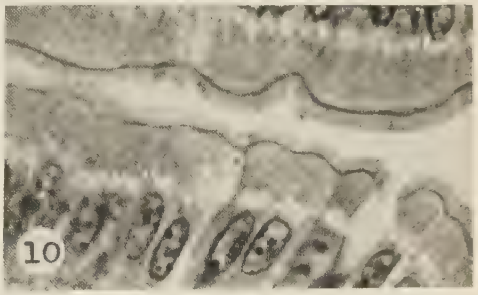
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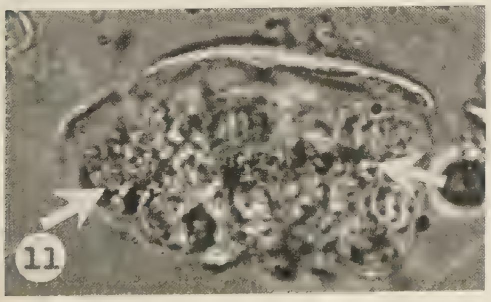
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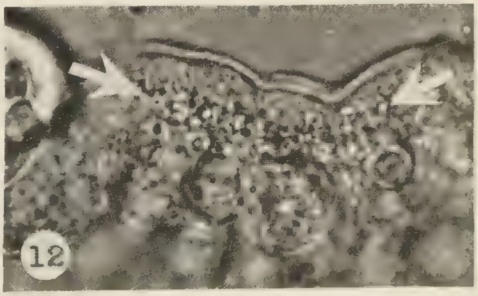
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Epinephrine and the Latency of Muscular Contraction[1]

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In etherized cats, Gruber ('24) reported a reduction by epinephrine chloride, 10^{-3} to 10^{-5}, of the latent period of the indirectly stimulated isometrically contracting tibialis anticus muscle, in both resting and fatigued states. At an initial tension of 70 gm, average overall latency values were 17 milliseconds before and 16 after epinephrine, no distinction being made amongst the components of the latent period. Nor were reductions evident in all experiments. For the small time intervals under consideration, the inertia of the lever must have been considerable and its natural frequency rather low; yet despite the handicap of instrumental lag, the kymograph records did bring out a small difference in the majority of the records.

In the present experiments, with the availability of faster recording methods, the effect upon nerve conduction plus junctional transmission time, muscle action potential, and true latency (here defined as the interval between the initiation of the muscle potential, and the abrupt external manifestation of tension) could be separated. Epinephrine was found to shorten the true latency, and this is interpreted as an effect of the hormone in enhancing the muscle's "active state" (Hill, '49). Such an influence is possible in addition to post-contraction biochemical effects reported and summarized e.g., by Ellis ('59) and Sutherland and Rall ('60) in their reviews, and is evident from the generally accepted rise in twitch tension. The increase in end plate potential (e.p.p.) (Krnjević and Miledi, '58a) likewise signals an effect of epinephrine on a process preceding the contraction.

Goffart and Ritchie ('52) concluded that adrenalin prolongs the active state and that this accounts for the increase in tension obtained in maximal twitches. Using the quick release technic, Edwards et al. ('56) observed that the quaternary ammonium cations TEA (tetraethylammonium) and TMA (tetramethylammonium), and choline prolong the active state, TEA being by far the most effective. Calcium and ouabain were found to have no effect on the active state.

The "latency relaxation" of Rauh ('22), Sandow ('41), and Abbott and Ritchie ('48), a phenomenon which precedes the external manifestation of shortening in the twitch, is of the order of 0.001 of the twitch tension, and begins about the middle of the latent period, has not been examined in relation to epinephrine. Both the "latency relaxation" and the early increase of transparency (D. K. Hill, '49) have been interpreted by Hill ('50) as "physical signs of the altered mechanical condition, presumably of an altered molecular pattern of the muscle proteins." This considerable alteration of mechanical state was made evident by the quick stretch experiments (Hill, '49). Beginning about half way through the latent period, the muscle rapidly becomes less extensible, and by the end of the latent period, manifests substantial resistance to stretch. The onset of the mechanical change (increase in rigidity, rapid decrease of extensibility) is simultaneous with the start of heat production. At this early stage, the heat production is presumably "heat of activation," and is viewed as a by-product of the chemical process by which the change in the muscle's mechanical state is effected.

METHODS

Dissection, fluid composition and construction of chamber. Female albino rats, 136–204 gm were killed by cervical fracture, to avoid complicating effects of the

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[1] Aided by grant B-1657 from the National Institutes of Health, Bethesda, Md.

barbiturate overdose technic, and a phrenic nerve-diaphragm preparation (muscle weight 51–101 mg, muscle width 12–14 mm) usually from the left side, was excised, with ribs attached, using a cold balanced salt solution during the dissection. Prior to opening the thoracic and peritoneal cavities, cold saline was injected to hasten cooling of the tissues. Composition of the balanced saline was NaCl, 9.0; KCl, 0.42; $CaCl_2$, 0.24; KH_2PO_4, 0.272; Na_2HPO_4, 0.20; $MgCl_2 \cdot 6H_2O$, 0.15 gm per liter distilled water. Glucose, 0.2%, was added immediately prior to use. At 25.5°C, the pH was 7.33. Sterling-Winthrop 1-epinephrine bitartrate was made up in 10^{-3} w/v concentration in distilled water, and kept at 5°C, before dilution during an experiment. Care was taken to incise the diaphragm parallel to the muscle fibers. The experiments were conducted at temperatures ranging from 27.2 to 29.7°C. For any given experiment, the variation was a fraction of a degree.

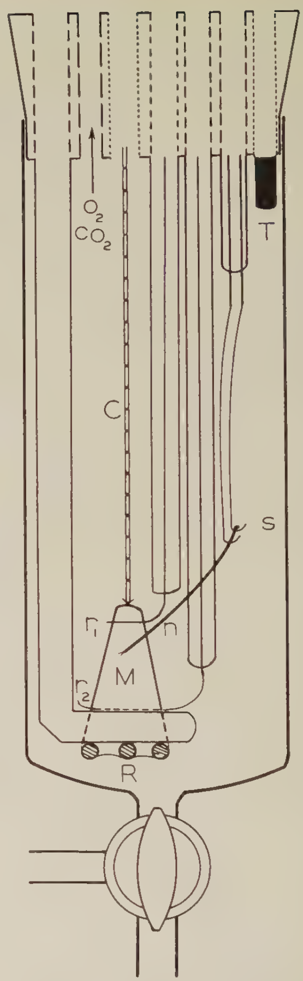

Fig. 1 Diagram of chamber for rat phrenic-diaphragm preparation. C, light chain connecting muscle to isometric lever and transducer. M, slip of diaphragm; n, phrenic nerve; O_2, CO_2, exit for gases perfused through chamber; r_1, r_2, external electrodes for recording muscle action potential. R, ribs (below glass slot); s, stimulating electrodes. T, thermometer bulb.

The nerve and muscle were threaded gently through the horizontal glass slot (1 mm wide) of the preparation mount (fig. 1) and a light gold-plated chain C (85 mm weighing 190 mg) tied to the central tendinous end of the muscle strip, which was fixed in position at one end by the ribs under the slot, and at the other end by attachment of the chain to the isometric lever. The chain was used to minimize elastic effects, such as may be introduced by a thread. For stimulation, the nerve was slung over the electrodes s, (no. 27 B. & S. gauge platinum wire) and with the aid of a dissecting loupe (2 × magnification) and fine watchmaker's forceps, tied lightly but snugly to each electrode, to prevent floating off during changing of the chamber fluid. Fluid was pipetted over the nerve and muscle repeatedly to prevent drying during attachment to the mount. Muscle action potentials were led off from platinum electrodes r_1 and r_2, r_2 lying in the slot on one side of the muscle, and r_1 approximately 11 mm above it, on the other side. The distance between the recording electrodes thus was kept constant in a given experiment. By covering the width of the excised muscle strip with the pair of electrodes, one on each side of the muscle, conditions for recording electrically from a major portion of the fibers were approached more nearly than is the case with a wire or other electrode of punctate configuration. The wires were sealed into the glass tubing by fusing the glass; and outside the chamber much finer plantinum wire was used for flexibility in coupling and removing the connectors from the electronic chassis, or coaxial cable. Tubing, rod, and thermometer T were fixed in position in a no. 7 rubber stopper, enabling ready removal of the entire mount from the chamber (32 mm I.D.). The stopcock was coupled to the chamber via translucent rubber tubing, not spliced directly. Although it requires more delicate manipulation, by mounting the nerve as described, the possible short-circuiting of the stimulating current by saline, which could occur in Bülbring's ('46) apparatus (where the nerve is drawn through a capillary) is avoided for the lines of current flow are now concentrated in the nerve; the entire nerve and muscle can be washed effectively; and provision is made for recording simultaneously the electrical activity and mechanical activity of the muscle in the same preparation.

Recording. Tension was recorded (fig. 2) by attaching the chain from the muscle in chamber C to the isometric lever L, which in turn actuated the plate shaft of the RCA 5734 mechano-electronic transducer, T. After 4-minute periods of immersion, the fluid was drawn off and tension and muscle potentials were recorded, in an atmosphere of oxygen, moistened by prior bubbling through saline.

Electronic equipment included an American Electronics precision stimulator, Grass A.C. and D.C. amplifiers, and Tektronix dual beam cathode ray oscilloscope. The traces were recorded on 35 mm film, with a camera attachment described elsewhere (Shapiro, '60). Twitch tension was recorded at a sweep speed of 1 cm in 50 milliseconds; electrical and mechanical latencies at sweep speeds of 1 cm in 1 and 5 milliseconds.

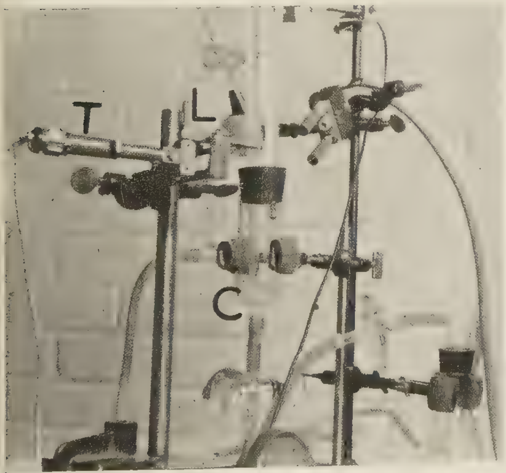

Fig. 2 Chamber and transducer. C, chamber; L, isometric lever coupled to T, transducer.

The initial tension was low, a few grams, and was adjusted by observation of the vertical deflection of the stationary spot of the oscilloscope. At the conclusion of each experiment, the muscle was cut away from the ribs and weighed, and a calibration of the lever carried out by momentarily suspending weights from the isometric lever, and recording the magnitude of the spot deflection. The calibration curves for spot deflection vs. load were of sigmoid shape. Voltage calibrations for muscle potentials were done before the experiment. The nerve was usually stimulated supramaximally with rectangular pulses, 0.1 milliseconds, 2.0 volts (cf. fig. 7).

Instrumental frequency response. By itself, and with suitable mechanical coupling, the plate shaft of the transducer tube is specified by the manufacturer to permit measurements of vibrations up to 12,000 c.p.s. As used in the present experiments, coupled to an isometric lever, the resonance frequency is reduced considerably below this figure. Following low twitch tensions, the lever manifested a low amplitude resonance vibration frequency of 220 c.p.s. This is adequate to discriminate the mechanical latencies of the order of 10 to 20 milliseconds, or more, dealt with in this study.

RESULTS

Twitch tension

In a typical run, the alterations in twitch tension are illustrated in figure 3. In (A), the gradual decline in tension occurring over a 21-minute period in saline was followed, (B), on application of epinephrine 10^{-6} w/v, by a restoration of tension, during a 24-minute period. This is in contrast to the finding reported by Bülbring ('46) that adrenaline increases the tension of the submaximal twitches but not that of the maximal. From examination of the film records of the present experiment, epinephrine did not induce repetitive firing of muscle action potentials; hence the epinephrine potentiation effect cannot be ascribed to brief muscle summations. On washout of epinephrine, (C), the tension falls back to the low values observed at the end of the control period (A).

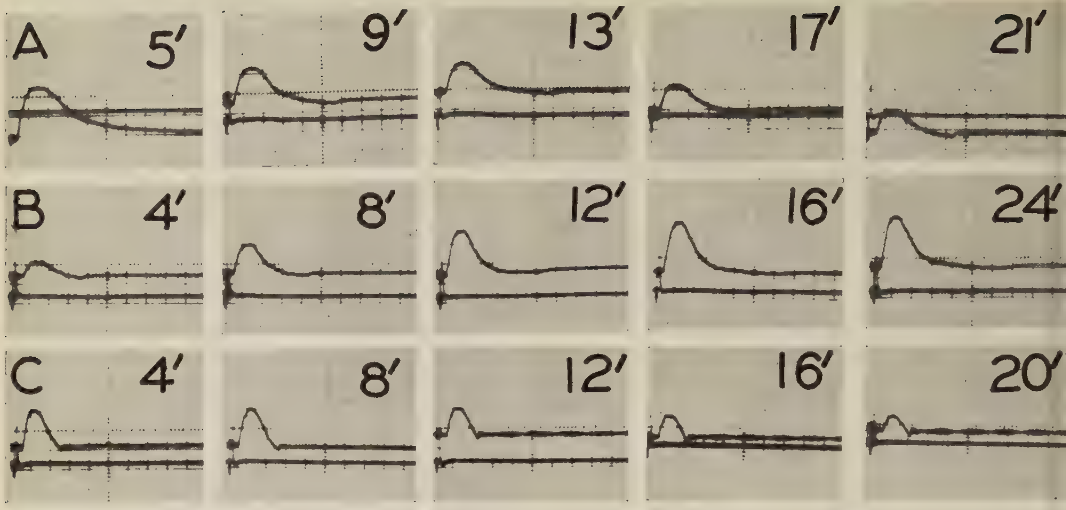

Fig. 3 Twitch tension (upper trace) and muscle action potential (lower trace). All records on same muscle. A, control after immersion in oxygenated balanced saline, for 5, 9, 13, 17 and 21 minutes, showing gradual decline in tension. Time base: 1 space = 50 milliseconds. B, enhancement of tension during immersion in 1-epinephrine bitartrate, 10^{-6}. Records at 4, 8, 12, 16, and 24 minutes. C, decline of tension during washout. Records at 4, 8, 12, 16, and 20 minutes; 27.6–28.1°C.

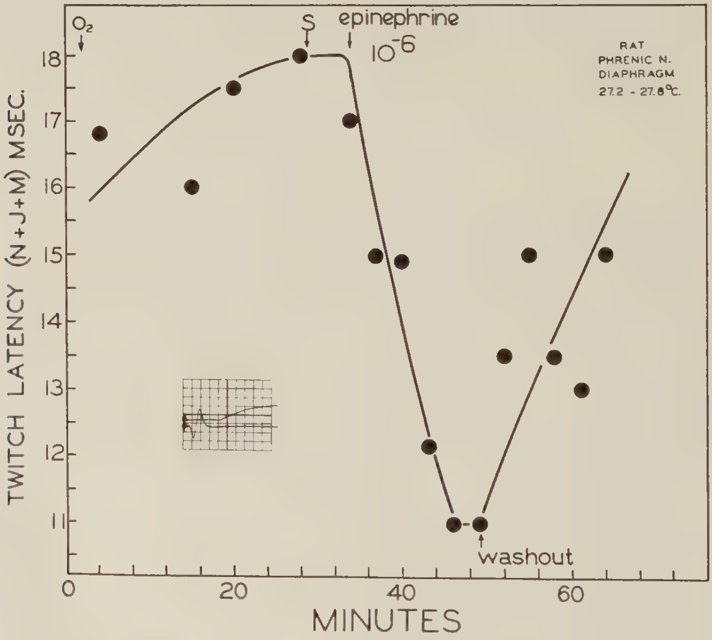

Fig. 4 Reduction in total twitch latency (nerve conduction plus n-m junction plus muscle) by epinephrine 10^{-6}, and reappearance of longer latencies on washout. Inset: Upper trace, total latency; lower trace, muscle action potential. Time base: 1 space = 5 milliseconds.

Total latency

To determine latencies, records of the type illustrated in the inset of figure 4 were made, with twitch tension above, and muscle action potential below. The end of the latent period is evident from the generally abrupt rise of the tension record. During the initial control period the tendency was for the total latency to lengthen, and to drop clearly on application of epinephrine. In the data shown, a slow control period lengthening of about 2 milliseconds was followed by a drop in latency from 17 to 11 milliseconds, with a subsequent rise on washout.

True muscle latency

To determine the true latency, the time from the initiation of the action potential, (which fires off as soon as the end plate potential exceeds a critical value) to the initiation of tension, was determined from measurements of records such as the inset of figure 4. The results from another preparation are plotted in figure 5. During the control period, when twitch tension dropped from 11.5 to 7.5 gm, the true latency lengthened slightly; epinephrine shortened the latency by about 4 milliseconds at the same time that twitch tension was restored to is original value. The time

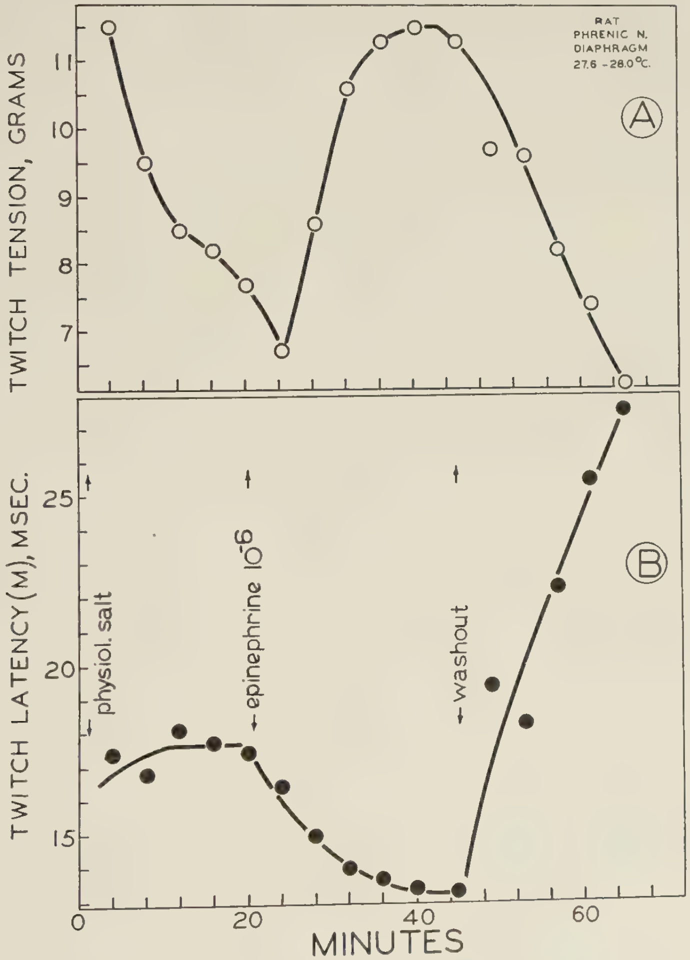

Fig. 5 Curves A and B from same muscle. Enhancement of twitch tension, and shortening of true muscle latency by epinephrine 10^{-6}. Reversals on washout.

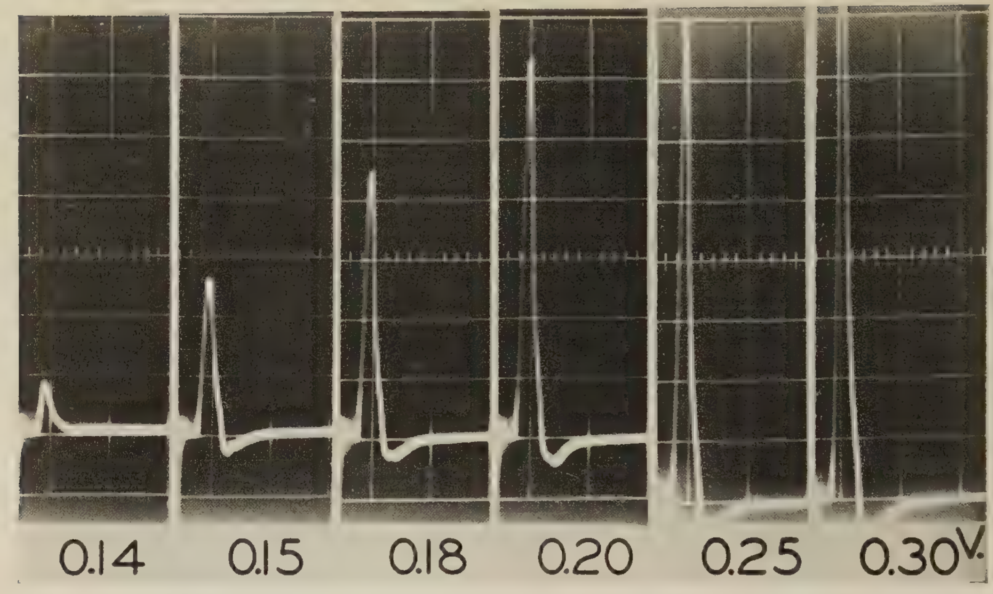

Fig. 6 Muscle action potential amplitudes in response to phrenic nerve stimulation of intensit indicated below each record, in volts.

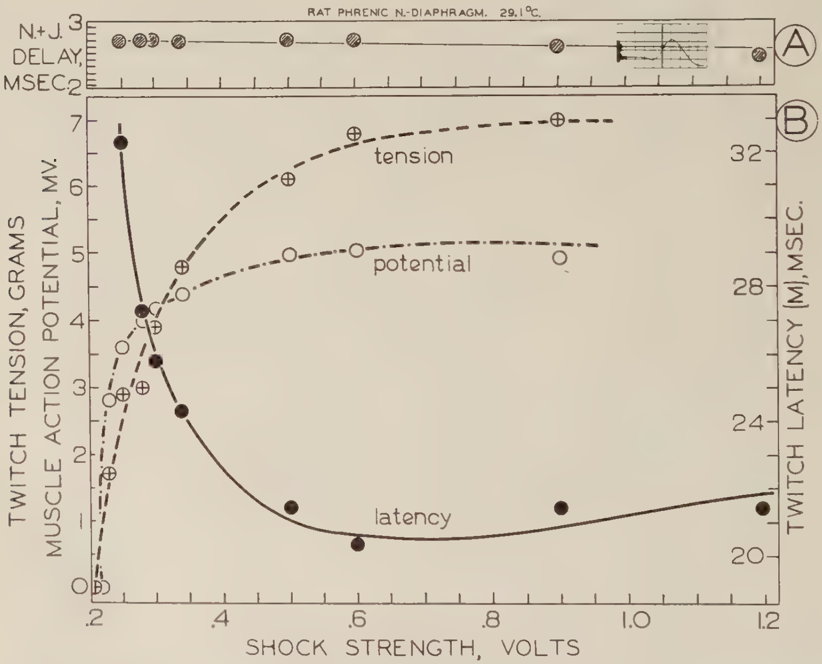

Fig. 7 Part A displays the relative constancy of delay due to nerve conduction plus junctiona transmission, from threshold to supramaximal shocks. Inset shows the initial deflections of the muscle action potential. Time base: 1 space = 1 millisecond. Part B, variation of twitch tension, muscl action potential, and true latency as a function of shock strength to phrenic nerve (from threshol to supramaximal). Data in A and B all obtained on the same preparation.

taken for epinephrine to exert its maximum effect represents presumably the kinetics of the diffusion process during which epinephrine passes from the solution to the surfaces of the innermost muscle fibers. Krnjević and Miledi ('58b) counted 12 to 24 layers of muscle fibers (aver. 17) in the rat diaphragm, and counts of this magnitude have been obtained in sections prepared in the present study. On washout, tension declined and latency rose progressively to a high value of 27 milliseconds.

Following the above experiment, after reversal by washout, the shortening of latency by epinephrine was demonstrated in a second run on the same muscle.

Effects of shock strength

1. *Muscle action potentials.* With increases in stimulation voltages applied to the nerve, the muscle action potentials of figure 6 are illustrative of the recruitment of motor units, thereby gradually increasing the externally recorded potential to a plateau.

2. *Nerve plus junctional delay* $(N + J)$. With fast sweeps, from records of the type illustrated in the inset of figure 7A, changes in shock strength to the nerve, from threshold to supramaximal had no significant effect upon $(N + J)$. Both tension and muscle potential rose monotonically to plateaus (fig. 7B), while the true latency

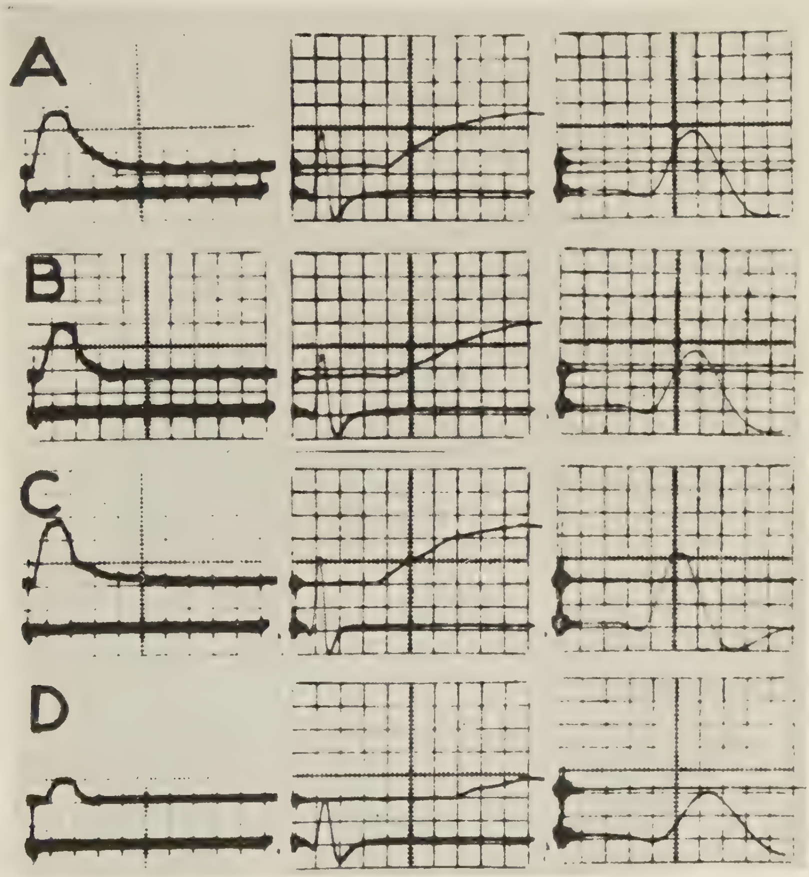

Fig. 8 In each record: upper trace, twitch tension; lower trace, muscle action potential. Time bases from left to right: 1 space = 50, 5 and 1 millisecond respectively. Left column, for twitch tension. Center, for total latency (nerve plus junction plus muscle). Right, (cf. fig. 7A) for nerve plus junctional latency. All records single sweeps, and from same preparation. A, control, in balanced saline, after 21 minutes immersion. B, the same, after 29 minutes. C, after 4 minutes in epinephrine 10^{-6}. D, after 26 minutes washout.

fell to a plateau. This information enabled the selection of a supramaximal stimulating voltage which would assure operation well over to the right, on the flat portions of these curves. Additional observations were made on excised phrenic nerve, to determine refractoriness, intensity-duration, and shock strength vs. action potential characteristics.

In figure 8 are displayed sets of records on the same preparation, at three sweep speeds, A and B in saline, C after epinephrine, and D, on washout. (N + J) and muscle latencies, tension, and muscle potential changes typical of a complete run appear together. From still another preparation, the complete graph of all the relevant variables are assembled in figure 9. During the measurements, over a period of about 100 minutes, the temperature rose from 28.6 to 29.1°C. The fluctuations of (N + J), figure 9B, occurring within a band of 0.3 millisecond, appear to be primarily of a random nature, and rule out the possibility that the observed effects are in any significant degree due to changes in transmission time. In other experiments, the variation in (N + J)

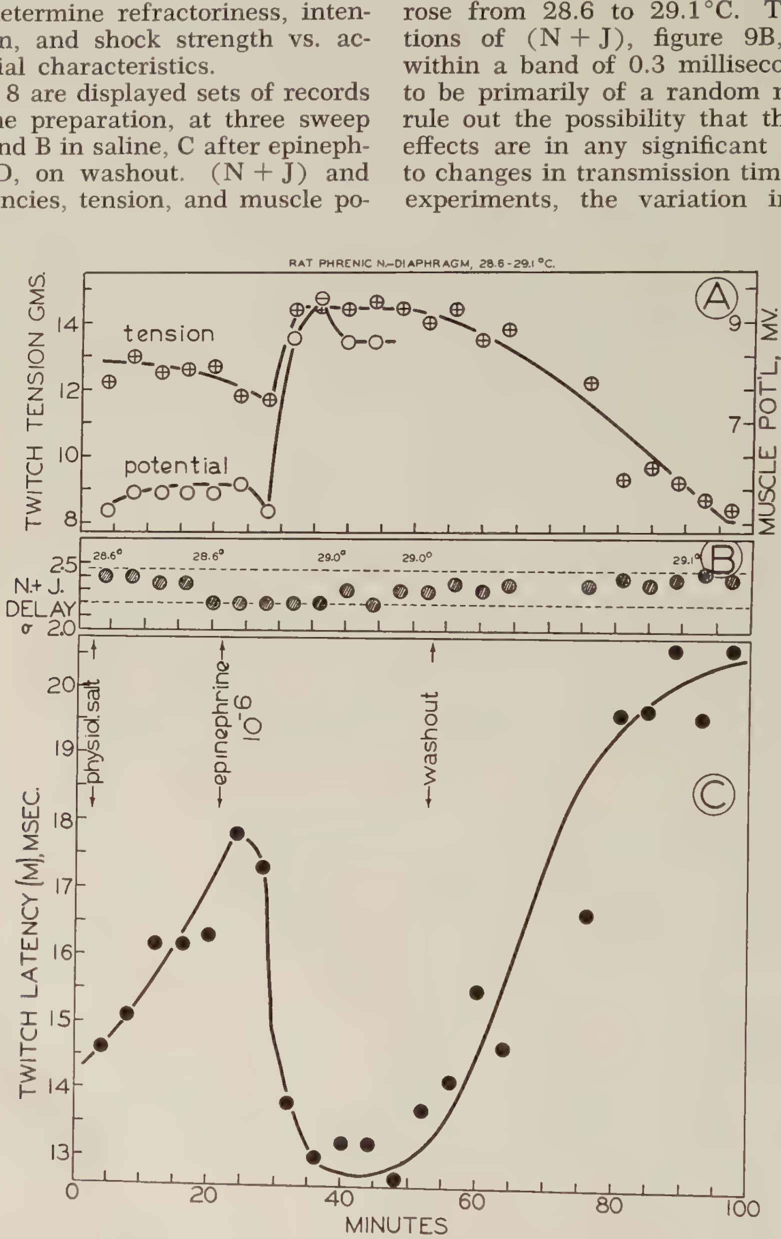

Fig. 9 Effect of epinephrine 10^{-6} on A, twitch tension and muscle action potential; B, nerve conduction plus junctional transmission; C, true muscle latency (from beginning of muscle action potential to first external manifestation of tension). All data from the same preparation. In B, total variation within a band of 0.3 millisecond.

atency was considerably less than 0.3 millisecond.

Tetani

Following several typical runs with epinephrine, when the twitch tension declined after washout, it was of interest to assess the viability of the preparation in oxygen, by applying brief tetani repeatedly over long periods. Figure 10 is illustrative of the findings. In a 26-minute period, 62 successive brief tetani, 60 shocks per second, (2 volts, 0.1 millisecond duration shocks) were applied to the nerve. Throughout this period, the muscle action potentials followed the rate at which the nerve was driven, and the complete tetanus tension which was elicited, did not fall to any considerable extent. This would indicate that no progressive failure of neuromuscular transmission was occurring, and that the reduction in twitch tension was due rather to downhill changes in the intensity and/or duration of the active state. On application of tetani, summation of responses could occur to give the observed steady tension responses.

DISCUSSION

Neuromuscular facilitation. The increase in peak muscle action potential upon addition of epinephrine, evident in figure 9A, raises the question of facilitation of transmission in the twitch response to a single volley. From the experiments of Brown et al. ('48) on the rat diaphragm, it is apparent that one cannot inject the argument that epinephrine acts in this preparation (as used in their and in the present experiments) by relieving neuromuscular block. Although absolute values of tension or potential were not given, they found the effect of epinephrine, 2.5×10^{-6}, (approx. 37.8°C) upon tension identical in indirect stimulation, with submaximal and maximal shocks. Moreover, when a freshly excised muscle was fatigued by maximal nerve stimulation at one to two per second, a parallel decline was observed in the twitch tension resulting from nerve excitation, and in the tension produced by direct stimulation of the muscle. They concluded that their experiments provided no evidence of neuromuscular block. Their experiments with partially curarized muscle did not yield an entirely unequiv-

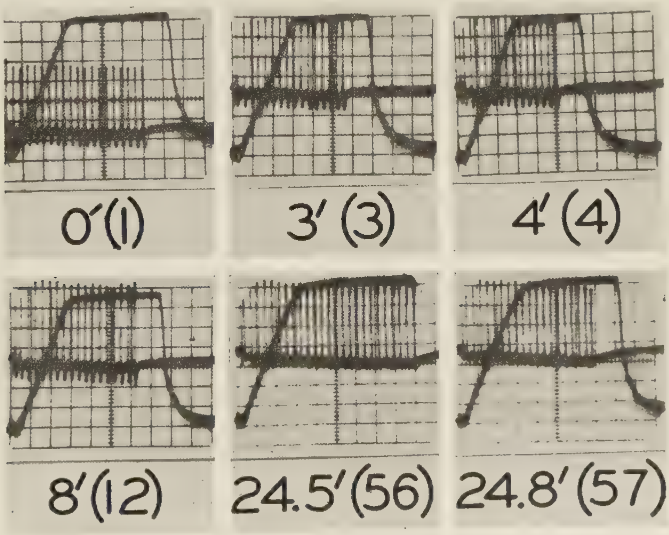

Fig. 10 Muscle action potentials and tension development in 57 brief tetani of the phrenic nerve. Supramaximal shocks, 60/sec. Upper row, first, third and 4th tetanus, at 0, 3, and 4 minutes. Lower row, 12th, 56th and 57th tetani, at 8, 24.5 and 24.8 minutes. This series of tetani was carried out subsequent to a regular run (as illustrated, e.g., in figs. 4, 5, and 9) to demonstrate that the muscle was capable of responding maximally, and that junctional transmission was unimpaired. Five additional tetani yielded same response. Time base: 1 space = 50 milliseconds; 29.3°C.

ocal answer, but did make it clear that whatever the effect of epinephrine upon junctional block, it is small compared with its direct action upon the muscle fiber. In a partially (40%) curarized maximally indirectly stimulated muscle, epinephrine 2.5×10^{-6} increased both action potential and muscle tension, the latter by a greater per cent.

In still other experiments on direct and indirect stimulation of cat soleus and tibialis anticus muscles, Brown and Burns ('49) concluded that the development of neuromuscular block is not responsible for the fall of tension which occurs as the system fatigues.

In the present experiments, the total nerve plus junctional transmission time was not significantly altered, again pointing to a minimal effect, if any, upon nerve and junction, for isolated twitch (non-repetitive) responses. Moreover, brief tetani over a period of one hour (fig. 10), when epinephrine may be presumed to have been almost completely oxidized at pH 7.3, gave no significantly reduced tetanic tension.

In the case of repetitive indirect stimulation of rat diaphragm, Krnjević and Miledi ('59) observed a presynaptic block of nerve conduction which may be intermittent or complete, but which is reversible if stimulation is discontinued or slowed. They consider that the presynaptic block is probably caused by anoxic changes in the intramuscular portion of the motor nerve.

Cat soleus muscle. Bowman and Zaimis ('58) have reported that epinephrine, norepinephrine and other sympathomimetic amines increase the maximal twitch tension of unfatigued skeletal cat muscles, but that the soleus muscle responded in a completely opposite way. It would be of interest to determine whether the latency changes due to epinephrine are likewise of opposite sign in soleus.

Parallelism between twitch tension and demarcation potential. Brown et al. ('50) observed that close arterial injections of epinephrine into the tibialis ant. muscle of the cat caused an increase of 2–15% in the tension response to single maximal nerve volleys, independently of muscular fatigue, as well as an increase of up to 10% in the demarcation potential of the muscle, which followed approximately th increase in twitch tension. These effect of the sympathomimetic amines were observed in denervated muscle and in muscl in which complete neuromuscular bloc was produced by d-tubocurarine chloride or by decamethonium iodide.

Krnjević and Miledi ('58a), using intracellular recording, were unable to observe any consistent change in resting potential after the addition of epinephrine but did demonstrate an increase in e.p.p (in 8 out of 15 trials).

Slowing of muscle impulse propagatio by epinephrine. Goffart and Ritchie ('52 demonstrated that the slowing of the propagated contraction wave along the muscl fiber (Brown et al., '48), induced by epinephrine, is not an important factor in th potentiation process. They showed this b determining that stimulation at one poin near the end of the muscle produced abou the same peak tension as all-over stimulation on a multi-electrode assembly.

Active state, velocity of shortening, an latent period in muscle. The pronounce diminution in muscle extensibility, which begins about half way through the laten period, marks the development of the "active state," which is defined as the maximum force which the muscle can bea without lengthening (Hill, '50). By th end of the latent period a considerabl resistance to stretch has developed. Th internal change from rest to activity i fully developed within 2% of the time normally occupied in a twitch. The form o the twitch depends on the velocity o shortening as a function of load, and thi is governed by the characteristic relation between force and speed (Hill, '38). Th maximum tension attained in an isometri twitch represents a balance between continuing shortening and oncoming relaxation. In a twitch, relaxation begins befor the contractile elements have had time t pull the elastic ones enough to give maximum tension.

The onset of the active state is indee rapid, and starts off at its maximum rate It is conceivable that this initial rate in th untreated muscle is not necessarily th highest attainable rate, but that it may b speeded up still more, or slowed down b the addition of suitable catalytic agents It is suggested that not only might epi nephrine prolong the active state (Goffart and Ritchie, '52) but that it may enhance the velocity of shortening as well, and in so acting, lead to a diminution in the mechanical muscle latency. The latter would be achieved in consequence of the contractile element pulling out the series compliance more rapidly, thus bringing the muscle elements more quickly to the point where external tension is manifest, i.e., where the abrupt take-off from the base line occurs in the record. Even if epinephrine did not prolong the active state it could in principle enhance twitch tension by increasing velocity of shortening. This in turn might be based on effects on contractile or elastic elements, or both. Alternatively, with unaltered shortening velocity of the contractile elements, a change in the compliance of the series elastic element might be effected so it could be stretched out more rapidly during the latent period. Muscular force will be apparent externally when the contractile element has shortened enough to raise the tension in the elastic element. Different treatments are known to alter the twitch tension without appreciable effect on the tetanic tension (Macpherson and Wilkie, '54); presumably this is achieved by altering the duration or intensity of the active state.

Within a certain range, hypotonic solutions enhance the twitch, whereas hypertonic solutions diminish, or even abolish the twitch of frog muscle, depending on the concentration (Howarth, '58). The velocity of shortening is reduced in hypertonic solutions; since part of the latent period represents time needed for the muscle to take up internal slack (contractile elements extending the elastic components), the latent period would be extended. Horwarth's experiments bore this out. In normal Ringer (1R) the latency of the frog sartorius at 0°C was about 13 milliseconds, and in 2.7R was prolonged to approximately 110 milliseconds. In solutions 2.5–3R, the twitch response to a single stimulus was abolished, yet the muscle action potential initiates activity in the contractile proteins. The failure to develop tension appears to be due to briefness of the active state, and the greatly retarded velocity of shortening, which fails to extend fully the series elastic compliance. The activation heat (Hill, '58) was essentially unchanged by hypertonic solutions even when the twitch was absent and there was no shortening heat or work.

Characteristic equation of Hill. Example of effect of chemical agent on velocity of shortening. The force-velocity relationships in muscle are describable quantitatively by the characteristic equation developed by Hill ('38) from combined thermal and mechanical measurements, viz., $(P + a)(V + b) = b(P_0 + a)$ where P represents the relatively steady tension in grams exerted during shortening at V cm/sec., a the heat of shortening per centimeter shortening, and b is a constant of proportionality representing the increase in rate of total extra energy liberation per gram decrease of load; b is also a measure of the speed of the shortening process, has a temperature coefficient of about 2.0 (Hill, '38) and an Arrhenius μ value of 13,000 calories (Shapiro, '39). In muscles exposed to carbon dioxide, the characteristic equation was found applicable and the value of b was reduced, irrespective of the temperature (Shapiro, '39b). This was interpreted as possibly due to shifting the pH to a less favorable region for the enzymes concerned. In the rat diaphragm, 5% CO_2 + 95% O_2 reduces the twitch tension (present series of experiments).

Excitation-contraction coupling. Sites of action of epinephrine. It should be pointed out that diffusion is too slow a process (Hill, '49) for transmission of activity from the muscle fiber surface to the interior where the shortening proteins are located. The nerve impulse may possibly play a role beyond that of a trigger setting off chemical events within the cell, by influencing reactivity, kinetics, or contractile protein states within the sarcoplasm according to the magnitude or rate of change of the electric potential. From an experimental and theoretical study of the effect of nitrate, iodide and bromide ion on frog sartorius muscles, Hill and Macpherson ('55) concluded that the active contractile state of muscle is initiated and maintained intracellularly by an influence transmitted from the excitable muscle membrane at the surface of the muscle fiber. The duration of the active state would depend on the kinetics of the initiating process at the surface. This they regard as a new link in the chain of events connecting excitation with contraction. In this context it is of interest to note that the e.p.p. is increased by epinephrine (Krnjević and Miledi, '58a; Shapiro, unpublished observations).

It is also conceivable that the milieu in which the contractile mechanism finds itself intracellularly influences the duration of the active state. Thus a substance might have two sites of action, one at the cell surface, and the other interiorly. Whether one or both are in effect playing a role may be determined by the type of analysis carried out by Hill and Macpherson ('55), and the experimental facts as elaborated by Kahn and Sandow ('50). In the present experiments, no such analysis has been carried out, but the relative promptness with which the epinephrine ion manifests its effects, and correspondingly the ease with which it is reversed on washout, point to a surface action. It is further possible that the active state may be affected intracellularly, and considerably beyond that, post contraction biochemistry, provided longer times are available, and oxidation has not taken place.

Furchgott et al. ('60) have observed that the increase in contractile strength of electrically driven guinea pig atria, mediated by epinephrine, was associated with an increase in the duration of the action potential.

Sandow ('52) Hill and Macpherson ('55), Gelfan ('58), Bourne ('60) and Huxley ('60) collectively have expounded current views on the mechanism of excitation-contraction coupling.

SUMMARY

1. The indirectly supramaximally stimulated rat phrenic nerve-diaphragm preparation, in oxygenated glucose-balanced salt solution at pH 7.3, was used in a specially devised chamber (figs. 1 and 2) for oscilloscopically recording twitch tension, muscle action potential through external electrodes, nerve conduction plus junctional transmission time, and true muscle latency (from the initiation of the muscle spike to the first external manifestation of mechanical shortening). The muscle was kept at a fixed length. Temperatures ranged from 27.2 to 29.7°C.

2. Nerve conduction plus junctiona transmission time (N + J), of the orde of 2.5 milliseconds, was not significantl affected by 1-epinephrine bitartrate, 10^{-} (fig. 9). The total maximum variation i the observed values of (N + J) encompassed approximately 0.3 millisecond This relative constancy was observed a submaximal as well as at maximal nerv stimulation.

3. At low initial tensions (a few grams) true mechanical latency was reduced (i the order of 5 milliseconds) by epinephrin 10^{-6} at the same time that tension an muscle action potential were enhance (figs. 5 and 9). On washout of epinephrine, the longer latencies reappeared, together with diminished twitch tension The natural vibration frequency of th transducer coupled to the isometric leve was measured and found adequate to demonstrate the mechanical latencies in th ranges observed.

4. The shortening of the mechnica latency is interpreted as due to an enhancement of those phases of the muscle's "active state" by epinephrine, by which the velocity of the contractile element can be accelerated so as to stretch th series compliance in a shorter time.

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The Pellicle as a Factor in the Stabilization of Cellular Form and Integrity: Effects of Externally Applied Enzymes on the Resistance of *Blepharisma* and *Paramecium* to Pressure-Induced Cytolysis

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Generally it is assumed that at least two structures are concerned with the maintenance of integrity and form in animal cells: an outermost membrane, or pellicle, and a peripheral layer of gelated cytoplasm, which here is considered to include the plasma membrane. The pellicle has been noted in a variety of cells, including protozoa, ova, blood corpuscles and ciliated epithelium (Chambers, '24) and the peripheral layer of gelated cytoplasm has been studied extensively (see Marsland, '56) since Mast ('26) first emphasized its importance in *Amoeba proteus*.

According to Chambers ('49), the pellicle is to be considered as an extraneous coat mainly of protein composition, secreted at the surface of the animal cell, and not completely essential to the continued life of the cell. Kudo ('54) regards it as a supporting structure, at least in reference to the protozoa. But among such forms the properties of the pellicle seem to vary considerably, according to species. In *Amoeba proteus,* it is thin and tenuous, while in *Amoeba verrucosa* it is exceptionally tough and may be stretched considerably with a microneedle without apparent injury to the cytoplasm beneath (Howland, '24). The pellicle of ciliates, on the other hand, is generally considered to be a relatively thick, tough, flexible layer, which helps to maintain the unique form of the different species. In *Paramecium* and *Stentor,* for example, it may be seen in the form of blister-like elevations, when these organisms are subjected to certain abnormal conditions. Merely compressing the animals under a cover-slip may produce such blisters, which collapse when pricked, without apparent injury to the cell (Kudo, '54). *Blepharisma,* moreover, may be induced to shed at least part of the pellicle when placed in 0.0001 M strychnine sulfate solution (Nadler, '29) or in dilute solutions of Janus green B (H. I. Hirshfield, personal demonstration). In such cases the pellicle remnant appears as a crumpled amorphous mass. The depelliculated blepharisma, on the other hand, display a definitely shorter less regular form which changes slightly from moment to moment as the animals continue to swim in a very sluggish manner. Moreover, such animals are extremely vulnerable to mechanical injury, and many disintegrate when pipetted from one medium to another.

The well-known solational action of high pressure on protoplasmic gels (see Marsland, '56) has made it possible to demonstrate the importance of the cortical gel layer in a variety of cells. However, not very much attention has been given to the ciliates since Regnard (1884) subjected *Paramecium* and related forms to pressures ranging up to 1000 atmospheres. Ebbecke ('35) and Hodapp and Luyet ('47) have reported that pressures in this range cause *Paramecium* to round up and undergo cytolysis. However, these workers were not able to observe the organisms during the compression and decompression periods. Consequently the precise nature of the effects could not be analyzed.

More recently, Auclair and Marsland ('58) studied the form-stability and cellular integrity of *Blepharisma* and *Paramecium,* directly observing the organisms in a

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microscope-compression chamber at pressures ranging up to 11,000 psi and at temperatures between 12 and 25°C. This work makes it clear that the pressure level required to cause the cilliates to lose form-stability with subsequent cytolysis showed a definite increase with increasing temperature. Auclair and Marsland, however, could not determine whether the primary effects were upon the pellicle or upon the subjacent plasmagel layer or upon both. To resolve this problem, accordingly, we have used the method of eroding the pellicular covering, either partially or completely, by enzymic digestion. Thus it has been possible to compare normal cells with those with eroded pellicles, with reference to their stability under various pressure conditions.

The principal enzymes used were trypsin and chymotrypsin, since these have proved very effective in removing the external coatings from various marine eggs (Allen, '57). However, hyaluronidase, alpha- and beta-amylase and beta-glucosidase were also tried, since the composition of the pellicle of ciliates is not very well-established.

MATERIAL AND METHODS

The ciliates, *Blepharisma undulans* (Suzuki, '54) and *Paramecium caudatum*, were used in this study because of their well-defined pellicle and their characteristic elongate shape, which tends to become ovoid when structural instability develops (Lewis, '42). Moreover, previous pressure studies on these organisms (Auclair and Marsland, '58) provided an excellent orientation for the present work.

Culture technique.

Test organisms were obtained as subcultures of mass cultures which have been maintained for many years at New York University. The ciliates were grown in Brandwein solution (Brandwein, '35) and kept in a darkened incubator at a temperature of 20–23°C. Periodically, single organisms were isolated and placed individually into the spots of a 12-spot Kline agglutination slide, where they were nourished by a lettuce-Brandwein infusion inoculated previously with the bacterium *Pseudomonas ovalis*, according to the method of H. I. Hirshfield (personal communication). After 5 days of growth in which the organisms divided approximately once each day (Hirshfield and Pecora, '56), the contents of the depressions containing the greatest numbers of normal organisms were transferred to test tubes of lettuce-*P. ovalis* medium and were allowed to incubate until well-established clones developed. These clones were found to give very consistent results when the enzyme and pressure experiments were performed on the 10th, 11th and 12th day following isolation. Organisms from younger or older clones tended to give more erratic responses and consequently only cloned animals of the designated age were used for the experiments.

Enzyme preparations

All enzymes employed were obtained from the Worthington Biochemical Company, Freehold, New Jersey and all were stored in a refrigerator before and during the period of use. Trypsin, beta-glucosidase and hyaluronidase were supplied in the crystalline, lyophilized state; the alpha- and beta-amylases were in solution. Fresh solutions of all but trypsin and chymotrypsin were prepared immediately prior to use and kept in the refrigerator. Concentrated solutions of trypsin and chymotrypsin were frozen in sterile 1-ml vials and kept in the freezer until needed. Then they were defrosted and used one at a time so that the entire stock solution was not denatured (Abderhalden and von Ehrenwall, '31, and Northrup, '32). The activity of solutions which had been frozen was checked periodically against that of freshly made solutions and was found to be comparable. All solutions were made up in working Brandwein solution, buffered to a pH near the activity optimum of the specific enzyme. The range in all the enzyme solutions tested was from pH 5.5 (0.1 M acetate buffer) to pH 7.6 (0.01 M phosphate buffer). Controls observed in Brandwein solutions buffered to these extremes showed no appreciable change in form, movement or sensitivity to pressure. Enzyme treatments were carried out in the individual spots of a Kline slide and the ciliates were washed carefully in Brandwein solution before treatment and between successive treatments. The ciliates were kept in a darkened moist-chamber at 25°C during the enzyme treatment. Then they were transferred to the pressure bomb.

Pressure techniques

The pressure bomb (Marsland, '50) permitted continuous observation of the pressurized specimens at magnifications up to 300 ×. At least 10 ciliates were used in each experiment and at least 10 experiments were performed for each enzyme treatment. The animals were contained within a small plastic cylinder (1.9 mm diam. × 1 mm height) placed at the center of the lower observation window of the pressure bomb, which kept them within working range of the microscope objective. In each case the enzyme treated specimens in the pressure chamber were allowed to equilibrate for 20 minutes at the experimental temperature, 20 ± 0.3°C, maintained by means of a constant temperature housing. The pressure, which ranged between 5000 and 8000 pounds per square inch (psi), was built up and maintained for 15 minutes. The pressure build-up time did not exceed 4 seconds and decompression, achieved by a release valve, was practically instantaneous. The organisms were observed and counted immediately before, and several times during the pressure treatment. Thus it was possible to ascertain the timing and the nature of the form changes and cytolysis reactions and to determine the percentage of cytolyzed individuals at the end of the 15-minute period. In all, between 100 and 200 ciliates were pressure-tested following each of the different enzyme treatments.

RESULTS

Enzyme treatment in general

As a basis for the pressure studies, first it was necessary to ascertain the action of a selected range of concentrations of the various enzyme solutions upon the immersed specimens. These preliminary tests made it possible to choose an appropriate concentration and treatment time, such that the enzyme effect would not be too slight to be detected in the subsequent pressure experiments, nor so severe that form-stability would be lost before pressurization. The tested concentrations ranged from 0.5% to 0.001% and the duration of treatment varied from 15 minutes to two hours. For the pressure experiments, minimal effective enzyme treatments were chosen and thus the elongate shape of the specimens was not lost as a criterion of form-stability under pressure (fig. 1A).

Blepharisma: effects of trypsin and chymotrypsin II

The effects of these two enzymes were so nearly identical that they may be considered together. *Blepharisma* exposed to a relatively high concentration (0.5%) of either of these proteolytic enzymes at a pH of 7.6 exhibited an immediate and extreme alteration of form and activity, although trypsin gave these effects at slightly lower concentrations or after shorter periods of exposure. All treated cells showed some effect within a few minutes, with a complete cessation of movement and a slight blunting of the ends (fig. 1B). Then the organisms gradually shortened, becoming wider and more ovoid (figs. 1C and 1D) until eventually they became completely round and many underwent cytolysis. Some of the animals attempted to pinch off the damaged portion of cytoplasm before complete lysis occurred and if successful, the pinched-off portion and the main cell body both became spherical and general cytolysis was prevented. However, only a very small number of the treated animals retained any degree of cellular integrity at these concentrations. But if the survivors were removed to fresh Brandwein solution after exposure to the enzyme, they became essentially normal in form and movement within 24 hours. Animals placed in lower concentrations recovered in a shorter time.

At intermediate concentrations (0.1%) and treatments of 30 minutes duration, approximately two-thirds of the specimens showed definite effects, while the remaining one-third appeared normal. The observed effects included a slowing or cessation of movement and the assumption of blunted, oval shapes.

Treated with more dilute solutions (0.05%), the blepharisma remained for the most part normal. Only 10–20% showed any abnormality of shape or behavior after

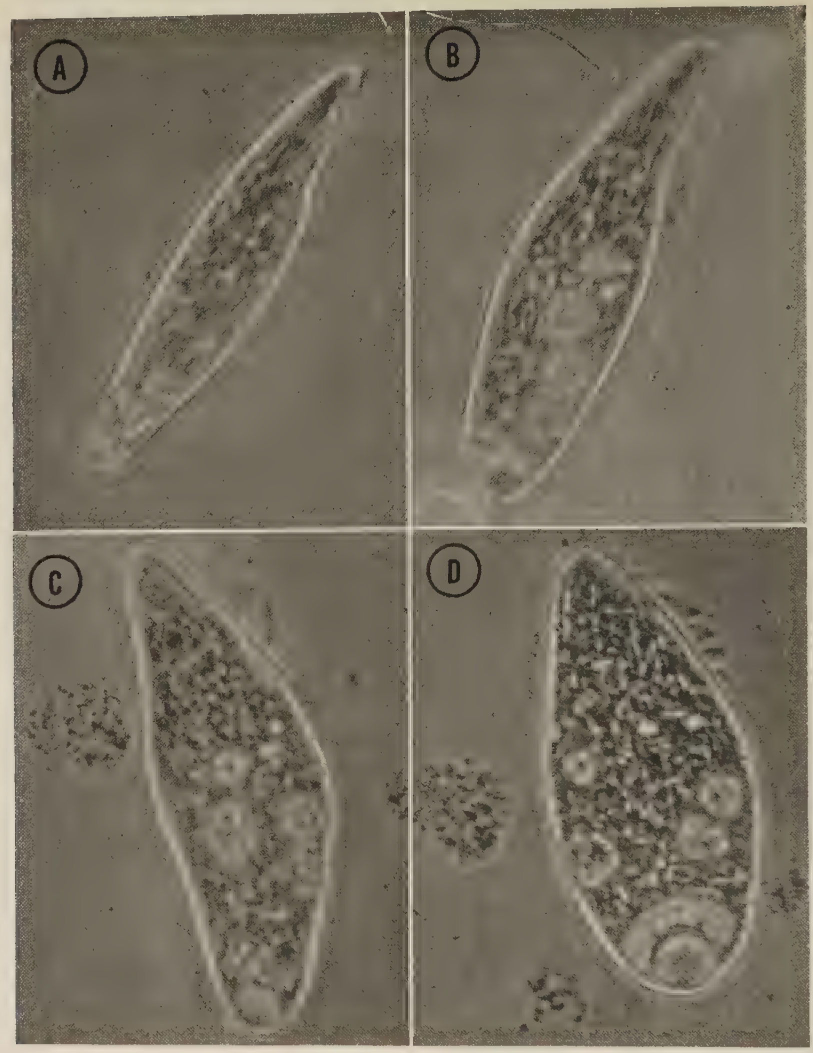

Fig. 1 *Blepharisma:* reaction to high concentrations of trypsin (0.5%, pH 7.8). A, normal specimen before addition of enzyme. B, specimen slightly blunted and shortened, photographed 30 seconds after start of enzyme treatment. C and D, 60 and 120 seconds later, showing morphological distortion and progressive rounding.

30 minutes in the protease. This percentage was lowered still further by employing solutions of 0.03%, in which 95% of the specimens appeared normal. This then was the concentration selected to precede the pressure treatment. However, all concentrations of trypsin and chymotrypsin produced a definite fragility in the cells. There was an obvious "stickiness" and tendency for them to adhere to the slide. Frequently it was necessary to use a gentle stream of fluid from the micropipette to dislodge the specimens for transfer, and often this procedure sufficed to cause lysis in some of the cells.

Blepharisma: increased sensitivity to pressure following trypsin and chymotrypsin treatment

The enzyme treated specimens were distinctly more susceptible to pressure-induced rounding and cytolysis than the untreated controls (see table 1). Form instability followed by lysis developed more quickly and at definitely lower levels of pressure. Thus the *critical pressure* (here defined as the minimal pressure yielding 50% cytolysis in 15 minutes at 20°C) was 7000 to 8000 psi, as compared to 9000 to 10,000 psi for untreated controls (see also Auclair and Marsland, '58). Moreover, virtually all of the cytolysis in the enzymized specimens occurred during the first 5 minutes of the compression period. Also the lysis was more abrupt and it occurred *without the preliminary formation of hyaline blisters*, so often observed in the control experiments. Usually the lysis began with a sudden rupturing of the cell surface, localized posteriorly near the contractile vacuole or anteriorly near the pointed extremity or in the peristomal region. Then the cytoplasm flowed out through the opening into the surrounding medium, where it broke up into small rounded droplets. Finally the cell remnants collapsed and slowly disintegrated. In all cases, a complete loss of motility and rounding of the cells preceded pressure-induced cytolysis. In general, lysis was more severe and complete in enzyme-treated cells as compared to untreated controls.

Rapid decompression regularly produced an abrupt further shortening of the partially rounded surviving cells, followed by lysis in a small percentage of cases. However, by far the major occurrence of lysis took place during the early part of the compression period. There was no immediate recovery of surviving cells following decompression. If transferred to plain Brandwein solution, many of the specimens did finally regain normal shape and movement within 2–3 days, although reproduction was not observed. A few animals seemed exceptionally resistant to the treatments, retaining a fairly normal appearance and behavior throughout.

Blepharisma: effects of hyaluronidase

The effects of hyaluronidase were very similar to those of trypsin and chymotrypsin. However, hyaluronidase produced these effects more rapidly and at slightly lower concentrations. Concentrations of 0.01% (at pH 5.5–6.9) caused an immediate change in the swimming pattern of the ciliates, although the morphological changes were slight. The animals tended to move sluggishly and often in a backward direction. At higher concentrations (0.03%), most of the exposed cells developed blunted ends and a generally shorter, wider form. Pressure testing showed that the form stability and cellular integrity of the blepharisma pre-treated with hy-

TABLE 1

Effects of various enzymes on the form-stability of Blepharisma undulans

Percentage of cytolysis induced by pressure applied after enzyme treatment. In each experiment the number of cytolyzed individuals was determined at the end of the compression period (15 minutes at 20°C).

Enzyme treatment	Duration of enzyme treatment	Pressure treatment	Cytolysis
	min.	*psi*	%
Controls (no enzymes)	—	7000	15
Controls (no enzymes)	—	8000	24
Controls (no enzymes)	—	9000	37
Trypsin (0.03%)	30	7000	55
Trypsin (boiled)	30	7000	11
Hyaluronidase (0.03%)	30	7000	58
Alpha-amylase (0.05%)	30	7000	18
Beta-amylase (0.05%)	30	7000	21
Trypsin + alpha-amylase	30 + 60	6000	24
Trypsin + alpha-amylase	30 + 60	7000	33
Trypsin + beta-amylase	30 + 60	7000	74
Trypsin + beta-glucosidate	30 + 60	7000	35
Trypsin + beta-amylase (boiled)	30 + 60	7000	58

aluronidase (0.03% at pH 5.5) was about the same as that of the trypsin or chymotrypsin treated animals (see table 1). The *critical pressure,* at which 50% of the specimens underwent cytolysis, was 7000 psi, i.e., 2000 psi lower than the control value. In most cases, also, the same type of cytolysis was observed. However, sometimes hyaline blisters appeared in the pressurized animals, in which case lysis occurred while the cells were attempting to pinch off the blisters and the cytoplasm escaped through a surface opening at the base of the blister. As in the previous experiments, following decompression there was no immediate recovery in the unlysed specimens, although they did regain normal appearance and motility after 2–3 days in fresh Brandwein solution.

Prior treatment with trypsin or chymotrypsin did not, on the other hand, augment the effects of a subsequent treatment with hyaluronidase.

Blepharisma: effects of alpha- and beta-amylase treatments

These two enzymes were also tried on blepharisma, first as initial treatments, and then following a preliminary exposure to trypsin. Even at high concentrations, initial treatments with alpha-amylase or beta-amylase produced little or no morphological alteration, aside from a very slight shortening of the cells after prolonged (24-hour) immersion in the enzyme. Furthermore, when these treated cells were subsequently exposed to high pressure, the form-stability and resistance to cytolysis was about the same as in untreated controls. However, when the alpha- and beta-amylases were employed following trypsin or chymotrypsin treatment, large differences appeared. Alpha-amylase had little augmenting effect after the proteolytic enzymes, while beta-amylase exerted a profound effect even at low concentrations.

Blepharisma, washed and placed in 0.03% *beta-amylase* after a 30-minute exposure to trypsin or chymotrypsin, underwent such severe reactions that many did not survive. At lower concentrations (0.01%) a better survival was obtained, even after a 60-minute exposure to the amylase. The animals appeared morphologically normal, although they were not very mobile and they displayed a pronounced stickiness. In the subsequent pressure tests, however, the form-stability and resistance to cytolysis was drastically reduced. A pressure of 7000 psi caused 70% lysis, in contrast to the usual 55% obtained when only the protease had been used. The cytolysis generally involved an abrupt disintegration of the entire cell, rather than a rupturing in a specific area as previously described. In some cases, the blepharisma abruptly folded in half as soon as the pressure was applied and the cytoplasm flowed out of a broad area on the convex side of the fold. Also, considerable additional lysis occurred during decompression and survivors exhibited no immediate recovery. As with other treatments, however, survivors of the enzyme-pressure treatment regained normal appearance and activity after several days in nutrient medium.

Blepharisma: effects of beta-glucosidase

Blepharisma treated solely with 0.05% beta-glucosidase solution for 30 minutes at pH 6.8 showed no sign of abnormality other than a slight decrease in motility. When such treatment was preceded by a 30-minute exposure to trypsin followed by a 10-minute wash in Brandwein solution, beta-glucosidase produced a number of blunted and rounded forms with reduced motility. No actual post-treatment lysis was seen, however, and in general the effects were much less striking than those caused by the combined trypsin and beta-amylase treatment. Moreover, these animals displayed an essentially normal resistance to pressure-induced form-instability and lysis. Under pressure, lysis of treated cells occurred gradually with the formation of typical hyaline blisters on the cell surface. Some animals continued swimming with these hyaline blisters and eventually succeeded in pinching off the affected material. Rapid decompression caused no further lysis and motility was usually regained by the decompressed blepharisma within 5 minutes. When the pH of the beta-glucosidase solution was lowered to 5.5, where a greater rate of activity might be expected, no greater effect was observed. Survival of the blepharisma after both enzyme treatments was practically 100%.

Blepharisma: control experiments

In all cases, two types of controls were run, using animals of the same clone as that of the experimental cells. One type involved pressurizing the cells immersed in Brandwein solution buffered in the same manner as the enzyme. In the other type, the pressurized cells were exposed to the identical enzyme solutions employed in the experiments, except that the solutions were first boiled to destroy enzymatic activity. Fluid lost in this process was made up by the accurate addition of distilled water, bringing the solutions back to original concentration. In experiments in which a second enzyme treatment was employed, only the second enzyme was inactivated. The conditions for the controls were precisely the same as for experimental animals, i.e., the same concentrations of enzymes, duration of treatments, pH, temperature and pressures were employed.

In the pressure tests, the various control specimens showed essentially the same degree of lysis (15 ± 2% at 7000 psi), except, of course, for the protease-treated controls, in which case no greater lysis was caused by subsequent treatments with any of the boiled carbohydrases.

Blepharisma: strychnine sulphate treatment

Blepharisma were induced to shed the pellicle by exposing them to dilute solutions of strychnine sulphate, according to the method of Nadler ('29). In this procedure, the drug was made up to 0.0001 M in Brandwein solution, and one drop of this solution was added to the blepharisma in the depression of a Kline slide. Some of the organisms responded immediately by detaching the pellicle first from the posterior part of the cell, trailing it from a point of attachment near the anterior end. Here the pellicle was seen as a thin filament of granular material, distinctly separate from the cell itself. The depelliculated cells, misshapen and rounded, were extremely sluggish in movement.

When subjected to compression at 7000 psi, the depelliculated organisms displayed 52% lysis, a value very close to that observed with the trypsin, chymotrypsin and hyaluronidase treatments. Pressure lysis involved a general and abrupt collapse of the cell, without preliminary formation of hyaline blisters.

It was not possible to find strychnine solutions of weaker strength which would avoid pellicle-shedding and yet give definite pressure effects. Furthermore, the drug depelliculation invariably proved lethal. None of the organisms remained alive after 24 hours, even when restored to nutrient medium immediately after depelliculation. In general, the same effects and drawbacks were also encountered with the Janus green B technique, mentioned earlier.

Paramecium: comparative observations

Although Paramecia displayed much less interclonal and age variability than Blepharisma, similar standardized procedures were followed: only individuals from young (10–14 days old) cloned cultures were used. The controls, pressurized at 7000 psi at 20°C gave a 20% lysis value and this pressure-temperature treatment was used in testing the enzyme effects. The more rapid movements of the paramecia, however, made it necessary to confine them within very shallow plastic chambers placed on the lower window of the pressure bomb. Compared to blepharisma, the paramecia displayed greater residual motility at the test pressures. The animals continued to swim in spiral fashion, at somewhat reduced speed, though most of the specimens were slightly rounded, or blunted, at the extremities. Rapid decompression caused an immediate, though slight, further shortening, but normal motility reappeared very quickly and normal form was reassumed in a few minutes in all but the most drastically affected individuals.

Trypsin treatments, in the concentration range which was very effective for blepharisma, had very little effect with reference to pressure-induced lysis and form-instability in paramecium. Paramecia, treated with 0.03% trypsin for 30 minutes at pH 7.8, yielded essentially the same percentage of pressure cytolysis as did untreated controls. The only effects of the enzyme seemed to be some change in the swimming pattern of the specimens and a slight pre-pressure blunting of the ends. The same was true for paramecia exposed to alpha-amylase and beta-glucosidase solutions. Even extremely high concentrations of these carbohydrases caused little change in morphology or behavior, although no pressure experiments were done in the high ranges. Comparison tests, using the same enzyme solutions on the two ciliates, showed that trypsin solutions capable of decreasing the form-stability of *Blepharisma* under pressure, had only the slightest effect on *Paramecium*. When the concentration was raised to 0.05%, which caused immediate rounding and cytolysis of blepharisma, the effects on paramecia were limited to a distinct shortening and broadening of the normally elongated form. The specimens became

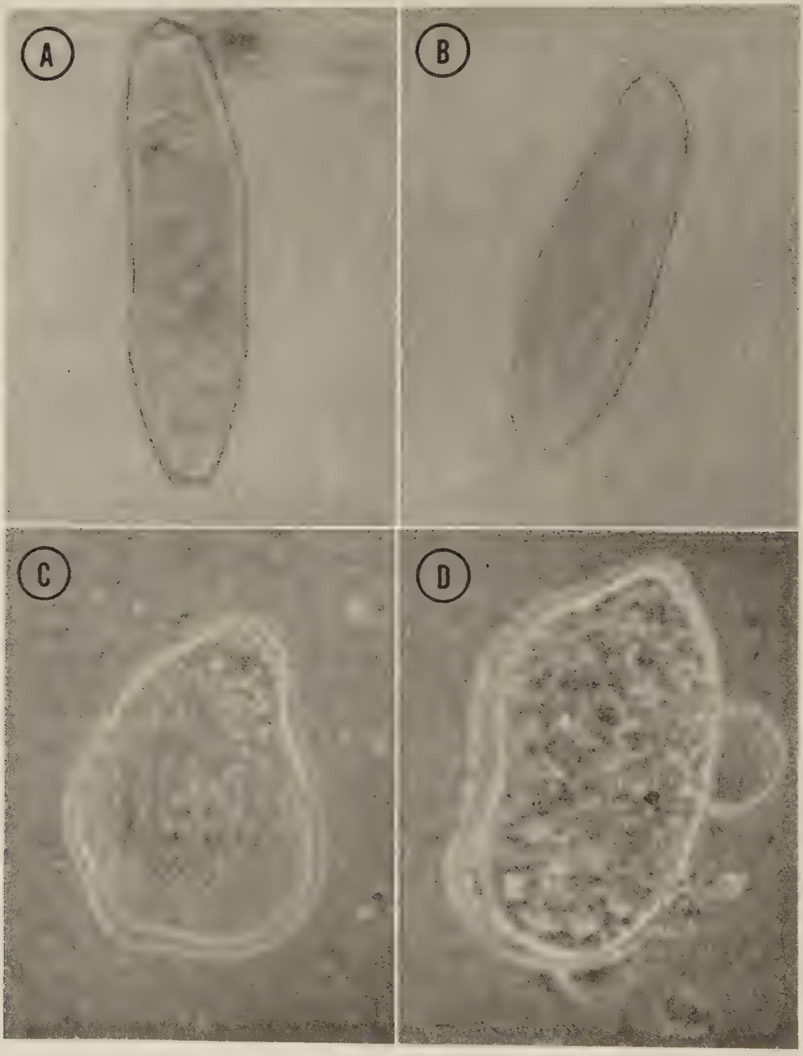

Fig. 2 *Paramecium:* reactions to various enzyme treatments. A, normal specimen, no treatment; B, beginning of shortening and blunting of extremities (hyaluronidase 0.05%, 30 minutes). C, rounding caused by beta-amylase (0.03%, 30 minutes); D, effects of extremely strong trypsin (1.0%) after 10 minutes exposure, showing rounding of cell and hyaline blisters along the periphery. (Outline of A and B retouched.)

very broad in the central region and with each extremity tapering rapidly to a blunt point. Such a "diamond-shaped" form did not provide a suitable criterion for testing the pressure-stability. In extremely strong solutions (1.0%) of the protease, the cytoplasm rapidly shrank away from the pellicle, which now appeared in the form of hyaline blisters around the periphery (fig. 2D). Paramecia treated in all but the 1.0% solution regained normal appearance and behavior after 48 hours in fresh Brandwein medium.

Paramecium showed some increase in the percentage of pressure cytolysis after exposure to 0.01% beta-amylase preparations (table 2). However, it was necessary to increase the duration of treatment to 60 minutes before a substantial pressure effect could be observed. This treatment gave a lysis value of 55% (at 7000 psi), which is significantly higher than the control value (20%). The treated animals were sluggish and slightly rounded before pressure and some showed an increased granulation of the cytoplasm. Lysis under pressure involved a rapid disintegration of the entire cell, beginning with a rupturing of the surface, usually in the peristome or near one end. At the onset of pressure, the paramecia suddenly became shorter, broader and more rounded at the extremities. Then the surface rupture developed, granular cytoplasm flowed out through the opening and complete lysis followed rapidly (figs. 3C and D). Decompression did not lead to further lysis, however, and the residual non-lysed individuals showed little, if any, recovery, as to motility and form, within the next 10 minutes.

There was some evidence that hyaluronidase had some tendency to reduce the form-stability of *Paramecium*, although the increase in pressure lysis was small. A pressure of 7000 psi after a 30-minute treatment in 0.05% hyaluronidase caused approximately 30% of the paramecia to cytolyze. Lysis occurred following a shortening and widening of the animal, particularly near the middle or peristomal regions. No decompression lysis was seen and the organisms soon regained motility, but not normal shape, after the release of pressure. Normal morphology was restored after several days, however, when the animals were transferred to Brandwein solutions.

DISCUSSION

The pellicle in relation to cell form and integrity

Although blepharisma and paramecium displayed distinctly different sensitivities to the various enzyme treatment, the evidence indicates that the pellicle, as well as the peripheral gelated cytoplasm, plays an essential role in maintaining cellular form and integrity in both species. In each case, structural stability, as tested by high pressure, showed a very definite reduction following short treatments with appropriate enzyme preparations. Moreover, complete rounding and cytolysis resulted from more prolonged exposures to the enzymes, even in the absence of high pressure. Presumably these effects resulted from a digestive erosion of the external covering, or pellicle, since it

TABLE 2

Enzyme-pressure studies on Paramecium caudatum

In each experiment, the number of cytolyzed specimens was determined at the end of the compression period (15 minutes at 20°C).

Enzyme treatment	Duration of enzyme treatment	Pressure treatment	Cytolysis
	min.	*psi*	%
Controls (no enzyme)	—	7000	20
Trypsin (0.03%)	30	7000	18
Trypsin (0.05%)	60	7000	18
Hyaluronidase (0.05%)	30	7000	29
Beta-amylase (0.01%)	60	7000	55

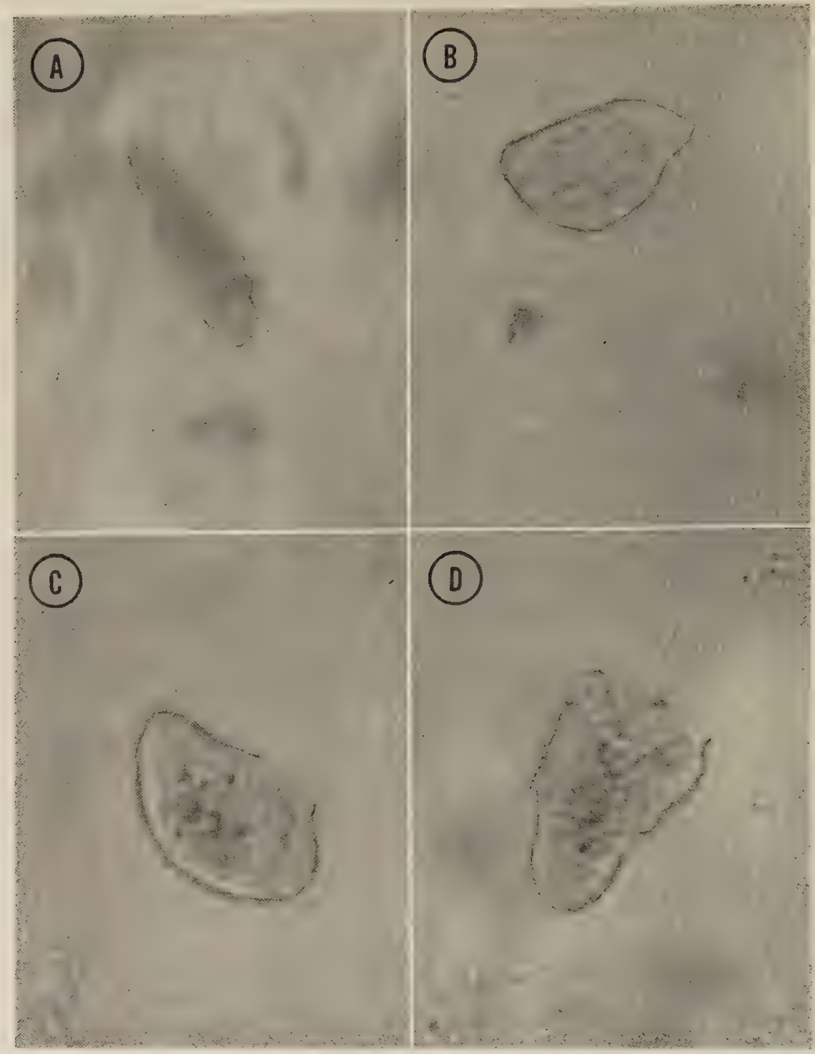

Fig. 3 Pressure effects on enzyme-treated ciliates. A, *Blepharisma,* previously treated with trypsin (0.03%, 30 minutes), one minute after the pressure build-up. The specimen is immobile and is beginning to shorten and widen. B, same specimen, 5 minutes later; severe rounding, cytolysis starting. C, *Paramecium:* one type of lysis induced by pressure (7000 psi) in a specimen previously treated with beta-amylase (0.01%, 60 minutes); lysis starting in region of the peristome. D, another type of lysis under same conditions; sudden disintegration of the whole posterior part of the specimen. (Outlines of A, B, C and D retouched.)

seems unlikely that the enzymes can penetrate through the true cytoplasmic surface, or plasma membrane (see Ballatine and Parpart, '40). Furthermore, the heat inactivated enzyme preparations proved altogether inert and usually the enzyme-induced cytolysis occurred without the formation of pellicular blisters. On the other hand, pressure alone, at somewhat higher levels, produced rounding and cytolysis. Here the primary action appears to be a weakening, or partial solation, of the cytoplasmic gel structure (see Marsland, '56, and Auclair and Marsland, '58). Thus it can be assumed that the pellicle and the peripheral gelated cytoplasm of these ciliated cells jointly stabilize the unique form and integrity of the species.

Special susceptibilities of the ciliates.

Blepharisma, *Paramecium* and many other ciliates, of course, display an exceptionally precise organization of form and structure in which the various organelles, cilia, peristome, trichocysts, etc., maintain a definitely fixed pattern of arrangement. Perhaps as might be expected, such cells seem to be unusually vulnerable to cytolysis when structurally weakened to a critical degree. Less fixedly organized cells, such as *Amoeba*, *Euglena* and even erythrocytes, in contrast, appear to be more stable, not only in reference to high pressure conditions, but also in reference to externally applied enzyme preparations. Thus Northrup ('26, '47) found that amoeba and euglena were not disrupted by trypsin applied at high (5.0%) concentration and for long (up to 6 hours) periods; and Parpart and Ballatine ('52) made similar observations on mammalian erythrocytes. Likewise, amoeba (Marsland, '56), euglena (Marsland, unpublished) and erythrocytes (Marsland, unpublished) do not undergo cytolysis when exposed to very high (up to 14,000 psi) pressures, although all of these cells do assume a spherical form as a result of the pressure treatment. In such more "mobile" cells, apparently, the maintainance of a fixed spatial interrelationship, especially with reference to peripherally localized structures, appears to be not so vital to the preservation of cellular integrity.

Differences between Blepharisma and Paramecium

Basically the different susceptibilities of the two species to externally applied enzymes may represent differences in accessability of appropriate sites of molecular structure upon which the activity of the enzymes may be focussed. Such differences on the molecular level naturally suggest that there may be morphological differences in the fine structure of the surface layers of the two species; and, indeed, this seems to be the case, as judged by the electronmicroscopy data which are available. The pellicular covering of Blepharisma, as observed by Dembitzer ('58), consists of a thin (15–20 A) dense outer layer superimposed upon a thick (200 A) inner layer of lesser density; whereas the pellicle of Paramecium (Sedar and Porter, '55) and a number of other ciliates (Reger and Beams, '54), consists of two layers of approximately equal thickness and density, together constituting a thickness of about 250 A. Moreover, the relation between the pellicle and the underlying protoplasm in different ciliates seems to be rather complex and somewhat variable (see Pitelka, '49 and Randall, '56, '57).

At present, however, it is not possible to find a very precise relationship between enzyme effectiveness and the molecular or morphological structure of the pellicle of ciliates, or of other cells. The positive action of hyaluronidase on both blepharisma and paramecium suggests the presence of one or more mucopolysaccharide components occupying a fairly accessible position in the pellicle. Also Monroy and Ruffo ('47) found hyaluronidase effective in removing the hyaline material which binds the blastomeres together in the developing *Arbacia* eggs, although Parpart et al. ('58) report that this enzyme acts primarily upon an inner layer of the hyaline material after an outer layer has been digested by alpha-amylase.

As to mucoproteins, which are considered to be represented quite widely among the components of the pellicular layers of various cells, the present evidence is somewhat ambivalent. The activity of trypsin and chymotrypsin so definitely observed in blepharisma but not in paramecium, suggests a species difference as to the position or orientation of the protein moiety in the pellicular architecture. A more superficial and accessible position of the molecular sites susceptible to proteolytic activity may obtain in the case of blepharisma, as also appears to be the case with the vitelline membrane of the echinoderm egg, which likewise is susceptible to digestive erosion by trypsin (Lindvall and Carsjo, '51). Moreover, the susceptibility of blepharisma to the action of alfa-amylase, applied subsequent to minimal treatments with trypsin or chymotrypsin, suggests that deeper sites in the pellicle structure have become available as a result of proteolytic erosion.

The relatively low activity of the proteolytic enzymes in the case of paramecium, on the other hand, cannot be taken to indicate that mucoproteins are absent from this pellicle. Fawcett and Porter ('54), using histochemical methods in their electron microscope studies on ciliated epithelial cells, have concluded that mucoproteins are very important in the pellicular structure and there is no reason to doubt that this may be true of many cells. Perhaps the pellicle of paramecium would prove susceptible to proteolytic activity following a preliminary treatment with hyaluronidase, but, unfortunately, time did not permit this approach to be included in the present series of experiments. On the other hand, the slight, but definite, activity of beta-amylase, in the case of paramecium, might indicate that the carbohydrate moiety of the mucoprotein component has a superficial localization, blocking off the sites of proteolytic activity, except when very high concentrations of the proteases are used.

SUMMARY

The resistance of the two ciliates to pressure-induced rounding and cytolysis was tested before and after exposure to various enzyme preparations (trypsin, chymotrypsin hyaluronidase, alfa- and beta-amylase, and beta-glucosidase).

Blepharisma displayed a particular sensitivity to the proteases and to hyaluronidase, whereas *Paramecium* was affected mainly by beta-amylase and hyaluronidase. Generally speaking, concentrated preparations of the enzymes induced rounding and cytolysis in a high percentage of the cells within one hour, even without the application of high pressure. Milder treatments, however, yielded specimens of essentially normal form and appearance which, none the less, proved to be less resistant to rounding and cytolysis when exposed to high pressures. Frequently the cytolysis of the enzyme-treated specimens occurred without the formation of pellicular blisters. Boiled enzyme preparations were altogether inactive.

These results are discussed in relation to species differences in the composition and fine morphology of the pellicular layers and to the role of the pellicle in stabilizing cellular form and integrity.

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The Structure of Slow and Fast Extrafusal Muscle Fibers in the Extraocular Muscles and their Nerve Endings in Guinea Pigs

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"Slow" extrafusal muscle fibers in the frog have been shown to have multiple "en grappe" motor terminations and to be of Felderstruktur type, while ordinary or twitch muscle fibers have a single "en plaque" ending and are Fibrillenstruktur in appearance (Krüger, '49; Günther, '49; Kuffler and Vaughan Williams, '53; Gray, '57, '58; Hess, '60b). Similarly, chicken and other avian extrafusal muscle fibers are "fast" and "slow," the former Fibrillenstruktur with a single "en plaque" ending, the latter Felderstruktur with multiple "en grappe" endings (Krüger and Günther, '58; Ginsborg, '60; Hess, '60c).

Krüger and Günther ('55) have claimed that mammalian muscles also can exhibit "fast" or "slow" muscle fibers with different kinds of nerve endings and a different internal structure. Krüger ('57) describes Felderstruktur and Fibrillenstruktur muscle fibers in the extraocular muscles. Most investigators have found "en grappe" terminations in mammals in the extraocular muscles only (see Tiegs, '53). While the present investigation was in progress, Kupfer ('60) found multiple endings on human extrafusal muscle fibers in the extraocular muscles. The multiple endings seen by Kupfer on intrafusal muscle fibers do not concern us here, and are the subject of another investigation (Hess, '60a); indeed, the guinea pig extraocular muscles, unlike the human, may be devoid of spindles.

The occurrence of multiple endings on mammalian extrafusal muscle fibers in extraocular muscles is confirmed in the present study. It is also attempted here to see if any structural differences can be found between mammalian extrafusal muscle fibers which receive multiple nerve endings and those which have only a single nerve ending.

MATERIAL AND METHODS

The extraocular muscles of guinea pigs were used. The muscles were fixed in an extended state in Susa or Dalton's fluid for one to two hours. They were then dehydrated, embedded in methacrylate, and thin and thick sections were made. The thick sections were used for the phase contrast microscope; the thin sections were photographed in the electron microscope. Some muscles were fixed in 10% glyoxal treated with calcium carbonate, teased, stained for cholinesterase by the Koelle method, and mounted in glycerine. Some of these muscle fibers, after identification of their nerve endings, were removed from the glycerine, refixed in Dalton's fluid for one hour, embedded in methacrylate, and viewed in the electron microscope.

RESULTS

All the observations enumerated below were found in all the extraocular muscles and apply equally to any one of the muscles.

Muscle fibers

Transverse sections of the muscle fibers fixed in Susa and viewed in the phase contrast microscope readily reveal two different kinds of muscle fiber (figs. 1A, B). In one, the cross-sections of fibrils yield a punctate appearance, and the fibrils appear separated by rather regular spaces from each other, while in the other, the fibrils appear larger and more irregular in shape and distribution. This corresponds to what has been seen in "fast" and "slow" muscle fibers of the frog and chicken. The punctate appearance is shown by twitch or "fast" muscle fibers of the Fibrillenstruktur type; the other appearance, called Felderstruktur, is shown by the "slow" extrafusal fibers of the frog and chicken.

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In transverse sections of the muscle fibers fixed in Dalton's fluid and viewed in the electron microscope, two kinds of fiber with differing internal structure can again be seen (figs. 3A, B). In one kind of fiber, the fibrils are discrete and separated from each other by regular spaces with each fibril surrounded by sarcoplasmic reticulum. This can be seen whether the section passes through the dark A or the light I band. The sarcoplasmic reticulum is more heavily concentrated around the I band. This apparently is the Fibrillenstruktur fiber (fig. 5A). The fibrils in the other type of fiber are not discrete. They are separated from each other by irregular spaces and their arrangement is more haphazard; each fibril is not surrounded by sarcoplasmic reticulum. Thus, several fibrils appear to touch or fuse together and are larger and not as orderly in arrangement as the other type of muscle fiber. This is the Felderstruktur muscle fiber (fig. 5B).

On the periphery of the extraocular muscles, several fibers occur which contain heavy concentrations of mitochondria in their borders and sometimes appear to be completely surrounded by mitochondria arranged roughly in two to 5 layers. These muscle fibers are thinner than those in the interior. Most of them present large fibrils in a rather disorderly arrangement and appear to be Felderstruktur in type (fig. 7B). However, there are some of these thin muscle fibers with dense mitochondrial accumulations on their borders which are of Fibrillenstruktur type (fig. 7A).

In every muscle, there are some muscle fibers that are not well fixed. These can show the fibrils very conspicuously with interfibrillar material missing. Although these muscle fibers are poorly fixed, they nevertheless serve to show the differences between the structure of Fibrillenstruktur and Felderstruktur muscle fibers perhaps even more markedly than well fixed fibers because the form and disposition of their fibrils are seen in even greater contrast. Some illustrations of these are presented (figs. 6A, B). The arrangement of the fibrils in the different muscle fiber types is as described above. These differences between kinds of muscle fiber are real and it is by no means intended to hint here that the differences between them are due to fixation artifacts. It is meant to point out that the differences between types of muscle fibers can still be seen in a variety of circumstances, even that of rather poor fixation.

In longitudinal sections fixed in Dalton's fluid and viewed in the phase contrast microscope, two kinds of muscle fiber can again be seen (fig. 2). In one, Fibrillenstruktur, the fibrils appear separated from each other and the Z-disk appears straight or smooth across the width of the fibrils, while in the other kind of fiber, Felderstruktur, the fibrils appear joined together and the Z-disk appears jagged.

In longitudinal sections fixed in Dalton's and viewed in the electron microscope, the differences between muscle fibers are again clearly seen (fig. 4). The fibers which apparently correspond to those of Fibrillenstruktur in transverse section again present an orderly arrangement of fibrils with each fibril surrounded by sarcoplasmic reticulum and separate from its neighbor. This is seen especially clearly at the level of the I band, where rather heavy accumulations of sarcoplasmic reticulum occur. In addition, the Z-disk across the width of the fibril is straight. In contrast, the fibrils of the Felderstruktur type of muscle fiber are not separated from each other, but appear to touch or join their neighbors at several points along their length. The sarcoplasmic reticulum is present, but does not completely surround each fibril. In addition, the Z-disk presents a jagged appearance and runs in a zig-zag fashion across the width of each fibril. This appearance corresponds almost exactly to the differences seen in longitudinal section between "fast" and "slow" muscle fibers of the frog and chicken.

Nerve endings

Morphology. Two kinds of nerve ending are revealed by cholinesterase staining. One ending stains darkly and appears as finger-like extensions encompassing a compact area of the muscle fiber with some of the extended processes interlacing. These endings are readily identifiable and appear like the motor end plates on the muscles of other mammals; these are the "en plaque" endings (figs. 11, 12, 16, 17).

The other kind of ending is very variable in shape and size and appears most frequently as a collection or series of droplets clustered on the muscle fiber (figs. 13A, B; 14). The endings are sometimes exquisitely delicate and extend by elongated branches for rather long distances on the muscle fiber (figs. 15; 18A, B; 19A, B; 20). This system can be rather elaborate and muscle fibers can be found which appear to be covered for a considerable distance by these delicate endings. At other times, muscle fibers are found on which the ending appears as a spot of cholinesterase-stained material (figs. 9A, B; 10). All these endings are probably of the "en grappe" type.

Distribution. "En plaque" endings and those of the "en grappe" type are never seen on the same muscle fiber.

The "en plaque" endings occur singly on an individual muscle fiber. The muscle fibers are about 35–40 μ in diameter. Muscle fibers were teased for distances of about 5 mm or more; many fibers were teased and two "en plaque" endings were never seen on the same muscle fiber.

Several "en grappe" endings were always seen on a single muscle fiber (figs. 9A, B; 10; 15; 18A, B; 19A, B; 20). The muscle fibers vary in diameter from 10 μ to 50 μ. Muscle fibers can vary in the distribution of "en grappe" endings from those appearing covered with nerve endings for relatively long distances (figs. 15; 18A, B; 19A, B; 20) to those on which the nerve endings appear as irregularly spaced spots (figs. 9A, B; 10). Very often, after the end of an extensive ending, the muscle fiber has no ending for a short distance, and then a shorter cluster of cholinesterase-stained material occurs. All variations of long elaborate endings and more compact clusters are found on the same muscle fiber. Also the muscle fiber can be devoid of endings for distances of 800 μ or more.

The so-called simplest "en grappe" endings, the spots of irregularly spaced cholinesterase-stained material, frequently occur on very thin muscle fibers (about 10–15 μ). Also, when they do occur on a muscle fiber, there are always several endings and they do not appear to alternate with the more extensive, elaborate "en grappe" endings, but rather to be more uniform in structure (figs. 9A, B; 10). It is suggested that these irregularly spaced spots occur on the thin muscle fibers described above which contain concentrations of mitochondria in their borders and which are located at the periphery of the extraocular muscles. Since these spots of cholinesterase-stained material occur irregularly spaced along the muscle fiber and do not appear to share a muscle fiber with the more elaborate "en grappe" endings, it would seem that the thin muscle fibers which have these simple "en grappe" endings do not have as many terminations and are less densely innervated than muscle fibers with more elaborate "en grappe" endings.

Correlation of kind of muscle fiber and kind of nerve ending

It is still necessary to attempt to correlate the kind of nerve ending, "en plaque" or "en grappe," with the kind of muscle fiber, Fibrillenstruktur or Felderstruktur. To do this, muscle fibers were fixed in glyoxal, stained for cholinesterase, teased, mounted in glycerine, and the kind of nerve ending identified. A bundle of about 8 to 10 muscle fibers, all with "en plaque" endings, was then rinsed in distilled water, refixed for one hour in Dalton's fluid, embedded in methacrylate, and viewed in the electron microscope. While it was possible to collect a group of muscle fibers, all with "en plaque" endings, it was found very difficult to procure a bundle of muscle fibers which had only "en grappe" endings. Thus, a small bundle of about 10 muscle fibers, consisting predominantly of muscle fibers with "en grappe" endings, always had two or three fibers with "en plaque" endings.

When a bundle of muscle fibers with all "en plaque" endings is cut in transverse section and examined in the electron microscope, the fibrils are discrete, separated from each other by fairly regular distances, and give the appearance of being Fibrillenstruktur in type (fig. 8A). All the muscle fibers in the bundle with only "en plaque" endings appear this way. In the mixed bundle of muscle fibers, predominantly with "en grappe" endings but with two or three "en plaque" endings, another kind of muscle fiber is seen in the electron microscope. Here, the fibrils are not distinct from each other, but rather seem to fuse or join together and are larger in appearance. This seems to indicate that these muscle fibers are Felderstruktur in type (fig. 8B). Most of the muscle fibers in the mixed bundle of predominantly "en grappe" endings were of Felderstruktur type, but probably corresponding to the muscle fibers with "en plaque" endings, some Fibrillenstruktur muscle fibers were seen.

Thus, from the above results, the conclusion seems strongly indicated that only "en plaque" endings occur on Fibrillenstruktur muscle fibers and "en grappe" endings occur on Felderstruktur muscle fibers only.

DISCUSSION

At least some mammalian extraocular muscle fibers thus conform to the morphological criteria established for "slow" muscle fibers in frogs and chickens. It remains to be seen whether muscle fibers in other mammalian muscles can be identified as "slow."

There is as yet a dearth of definitive work on the physiology of the extraocular muscle fibers in this regard. Acetylcholine produces a contracture, not a twitch, of the extrinsic eye muscles of the dog (Duke-Elder and Duke-Elder, '30); this also occurs with the "slow" muscles of other animals. The other physiological procedures necessary to identify "slow" muscle fibers, such as recording from single muscle fibers with intracellular electrodes or selective stimulation of small-motor nerves which innervate this type of fiber, remains to be done.

It has been suggested that perhaps the density of innervation of a muscle fiber determines whether a muscle fiber is "fast" or "slow" or in between these two extremes (see Hess, '60c). Thus, the multiply innervated fibers of the frog cannot be made to undergo a contraction or propagate a potential, while those of the chick, while ordinarily undergoing contracture, can be made to propagate a potential (Ginsborg, '60). This might be related to the observation that frog "en grappe" endings are more numerous and can occur closer together than those of the chick which occur at rather regular intervals. Similarly, mammalian intrafusal fibers, whose multiple endings are restricted to a relatively short stretch of the length of the muscle fiber (Hess, '60a), have the physiological characteristics of a "fast" muscle fiber rather than a "slow" one (Eyzaguirre, '60) If this be so and a relation does exist between density or frequency of occurrence of nerve endings and physiological properties of a muscle fiber, then perhaps it may be that there are three kinds of extrafusal fibers in the eye muscles: (1) "fast" fibers with a single "en plaque" ending; (2) "slow" fibers with several "en grappe" endings extending for long distances and at times practically covering the muscle fiber for long distances; and (3) muscle fibers with physiological characteristics neither typically "fast" or "slow" with simple "en grappe" endings not as numerous or extensive as the "slow" fiber, but rather scattered or irregularly spaced along the length of the muscle fiber. This third kind of muscle fiber may be the thin muscle fibers which have concentrations of mitochondria on their borders and which are located on the periphery of the extraocular muscles.

SUMMARY AND CONCLUSIONS

1. Extrafusal muscle fibers of the extrinsic muscles of the eye of the guinea pig were studied in the phase contrast and electron microscopes; their nerve endings were studied in teased preparations after cholinesterase staining. The observations enumerated below are seen in all the extraocular muscles.

2. Two kinds of extrafusal muscle fiber occur in the extraocular muscles of the guinea pig: Fibrillenstruktur and Felderstruktur. The Fibrillenstruktur fiber has each fibril surrounded by sarcoplasmic reticulum, the fibrils have an orderly ar

rangement with regular spaces between them and yield a punctate appearance in transverse sections, and the Z-disk runs straight across the width of the fibril in longitudinal sections. The Felderstruktur muscle fiber has fibrils which appear to join or anastomose with their neighbors, they are larger and more irregular in form and disposition, and the Z-disk presents a jagged appearance across the width of the fibril.

3. Thin muscle fibers with concentrations of mitochondria on the borders of the fiber are found on the periphery of extraocular muscles. Most of these fibers are Felderstruktur, some are Fibrillenstruktur.

4. Two kinds of nerve endings are seen after cholinesterase staining: "en plaque" and "en grappe." The "en plaque" endings have interlacing finger-like processes on a compact portion of the muscle fiber. The "en grappe" endings are seen as clusters or collections of droplets of stained material and are indeed "grape-like" or can appear simply as a darkly stained spot of material on the muscle fiber.

5. "En plaque" and "en grappe" endings never occur on the same muscle fiber. Only one "en plaque" ending occurs on a single muscle fiber; several "en grappe" endings occur on an individual muscle fiber. The spots of stained material occur irregularly spaced from each other; the more delicate clusters of droplets cover extensive lengths of muscle fibers and appear on some stretches of muscle fiber to be practically continuous from one ending to the next.

6. After cholinesterase staining, identification of nerve endings, refixation and embedding, and viewing the muscle fibers in the electron microscope, it was determined directly in the same preparation that "en plaque" endings occur only on Fibrillenstruktur muscle fibers and "en grappe" endings occur only on Felderstruktur muscle fibers.

7. It is suggested that the "en grappe" endings appearing as irregularly spaced spots occur on the thin Felderstruktur muscle fibers which have concentrations of mitochondria in their borders and which are located on the periphery of the extraocular muscles, while the more delicate and extensive "en grappe" endings occur on the other larger Felderstruktur fibers in the interior of the muscle.

8. Lastly, it is further suggested that the membrane characteristics of the muscle fibers may differ according to the distribution of their endings, and the fibers with an "en plaque" ending are "fast" fibers, the fibers extensively covered with "en grappe" endings are "slow" fibers, and the fibers with irregularly spaced simple spot "en grappe" endings are perhaps somewhere in between these two and not typically "fast" or "slow."

ACKNOWLEDGMENTS

The author wishes to express his deep appreciation to Mrs. Dorothy Sanderson for her capable and willing technical assistance and to Mr. M. W. Rhoades for his help in photography. This investigation was supported in part by research grants B-1487 and B-425 from the National Institute of Neurological Diseases and Blindness, United States Public Health Service.

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PLATE 1

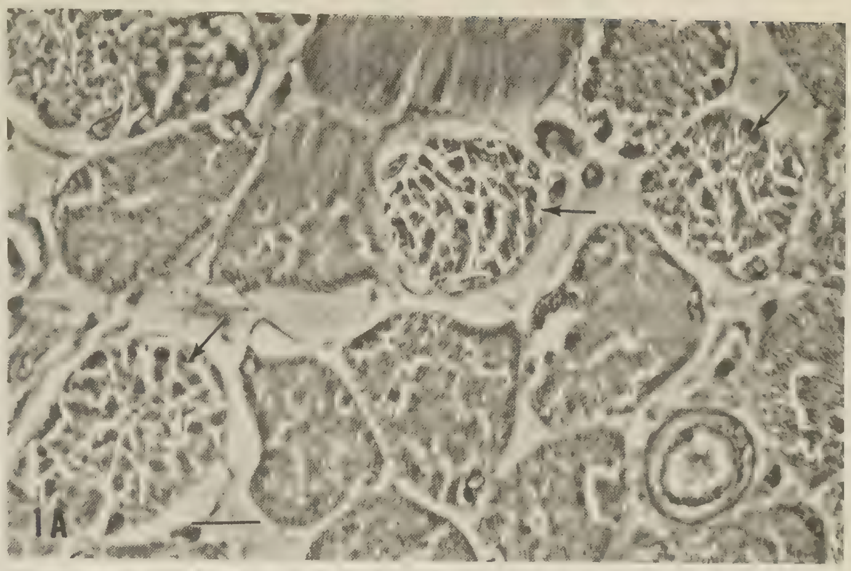
EXPLANATION OF FIGURES

Extraocular muscles, fixed in Susa, embedded in methacrylate, phase contrast micrographs. The lines on the photographs indicate 10 μ.

1A Transverse section. Some Felderstruktur muscle fibers are seen at the arrows, other muscle fibers are Fibrillenstruktur.

1B Transverse section. Felderstruktur muscle fiber on the left, other fibers are Fibrillenstruktur.

2 Longitudinal section. Felderstruktur muscle fiber at the top, Fibrillenstruktur muscle fiber at the bottom.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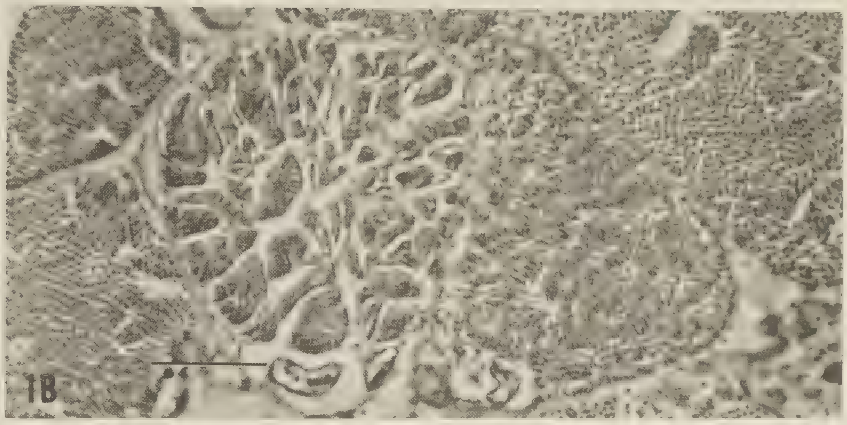
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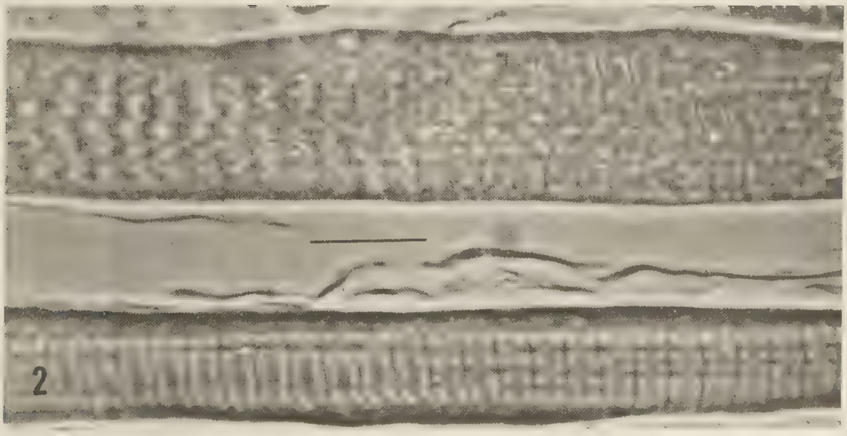
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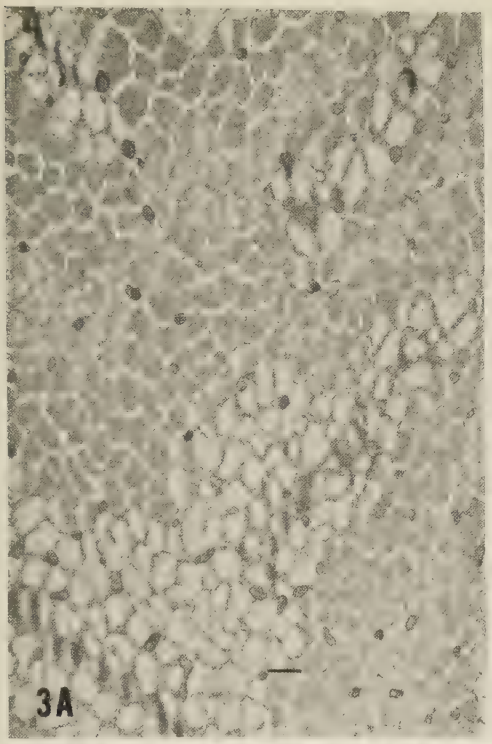
PLATE 2

EXPLANATION OF FIGURES

Extraocular muscles, fixed in Dalton, embedded in methacrylate, electron micrographs. The lines on the photographs indicate 1 μ.

3 Transverse section. A, Fibrillenstruktur; B, Felderstruktur.

4 Longitudinal section. Fibrillenstruktur muscle fiber on the left; Felderstruktur muscle fiber on the right.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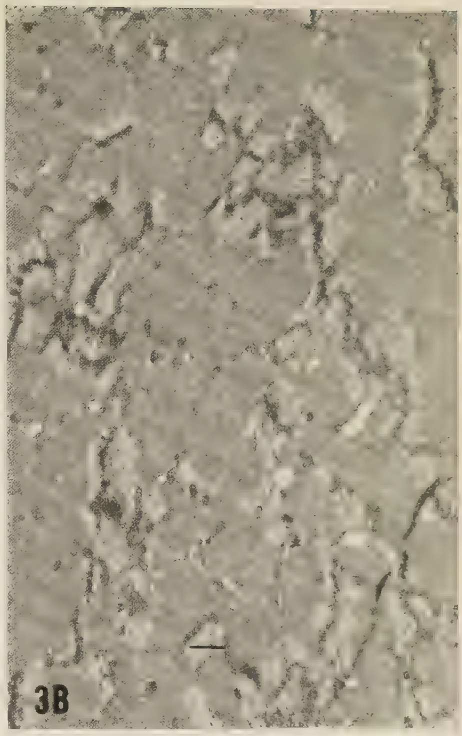
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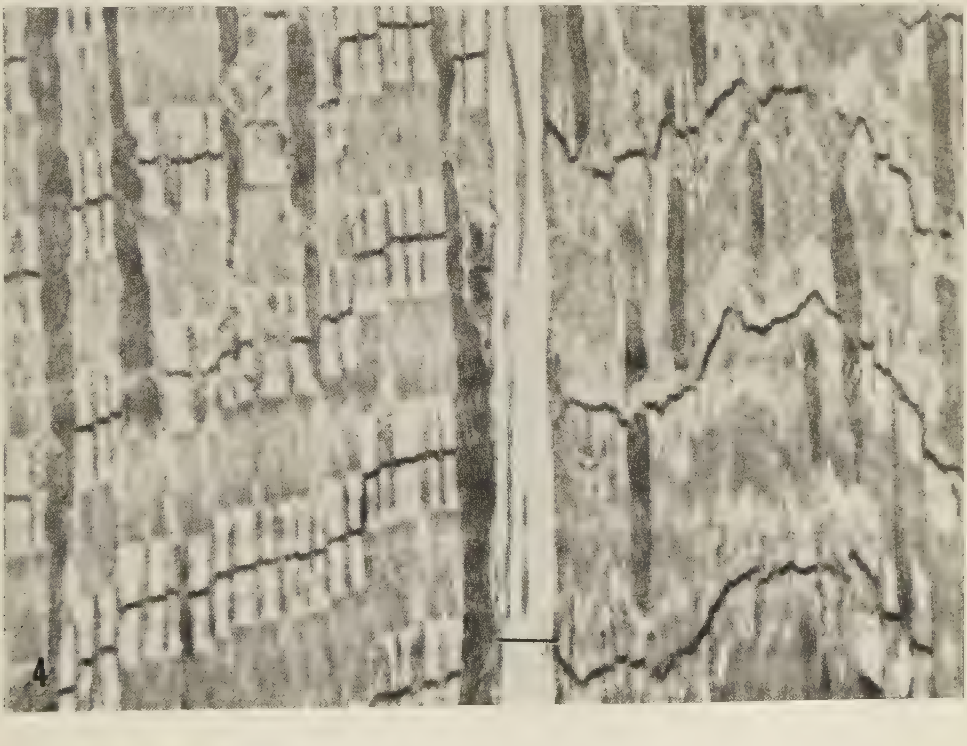
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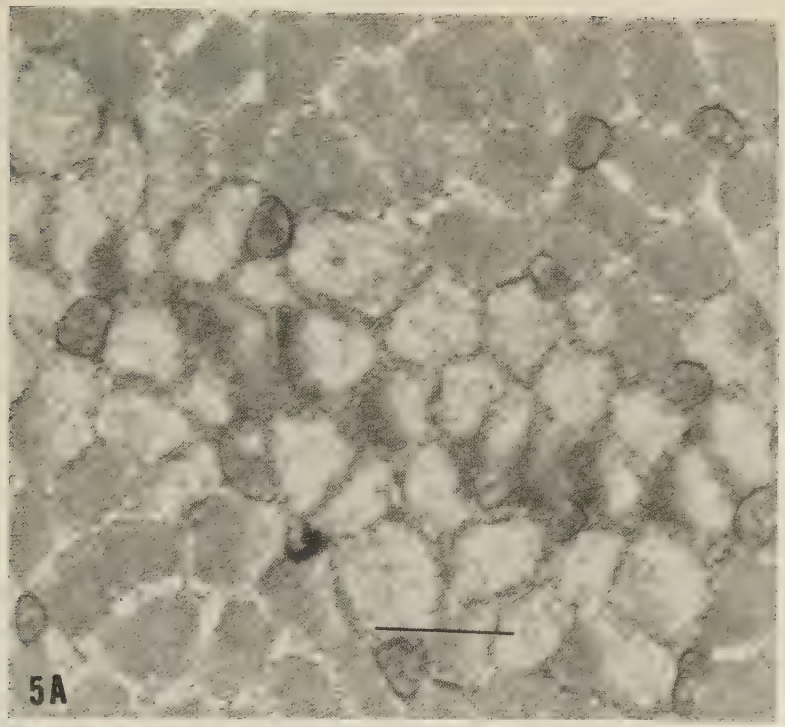
PLATE 3

EXPLANATION OF FIGURES

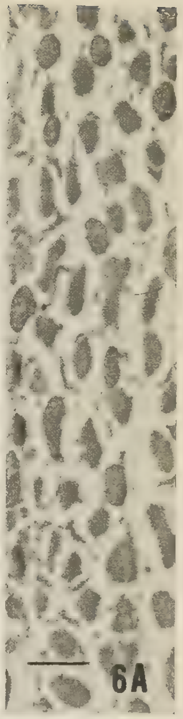
Extraocular muscles, fixed in Dalton, embedded in methacrylate, electron micrographs. The lines on the photographs indicate 1 μ.

5 Transverse sections. A, Fibrillenstruktur; B, Felderstruktur.

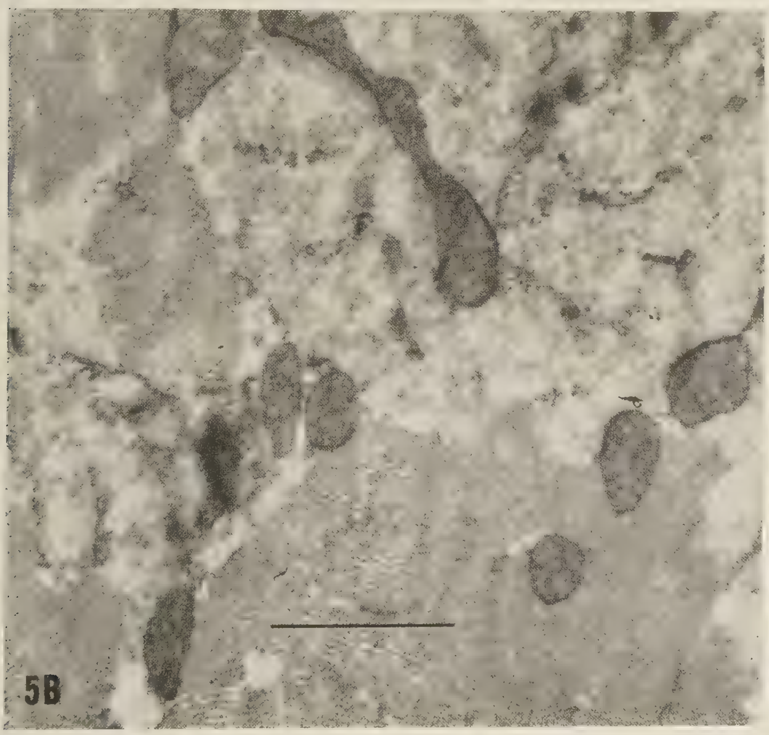
6 Transverse section of poorly fixed muscle fibers. Interfibrillar material missing. A, Fibrillenstruktur; B, Felderstruktur.



5A



6A



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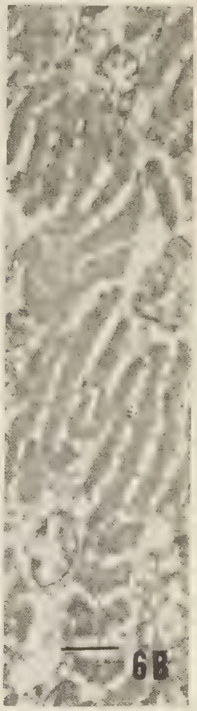


6B

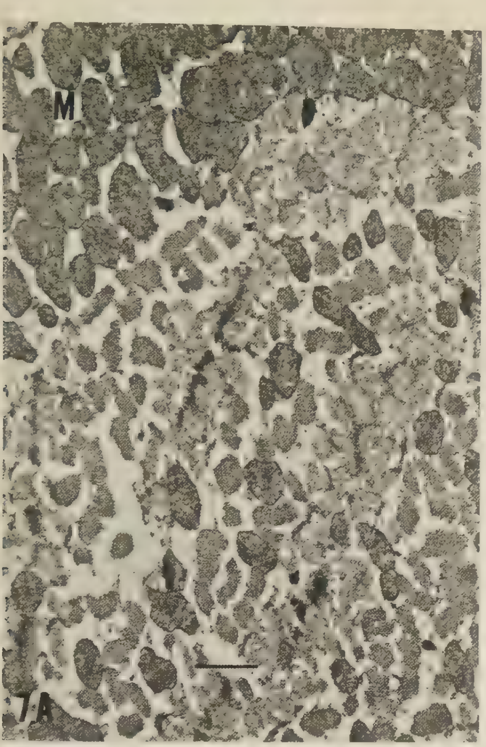
PLATE 4

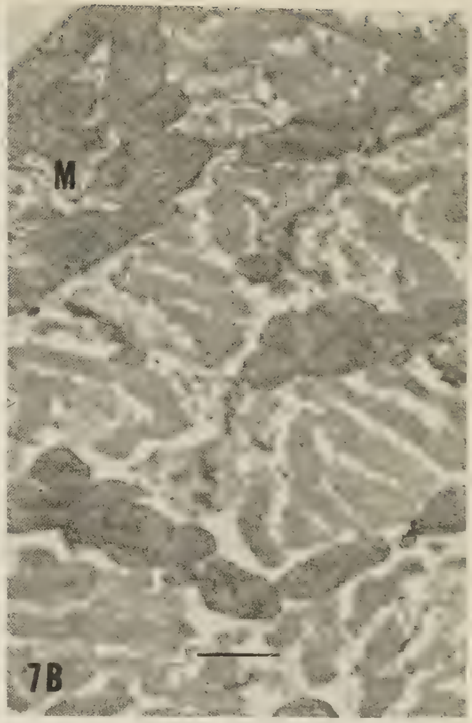
EXPLANATION OF FIGURES

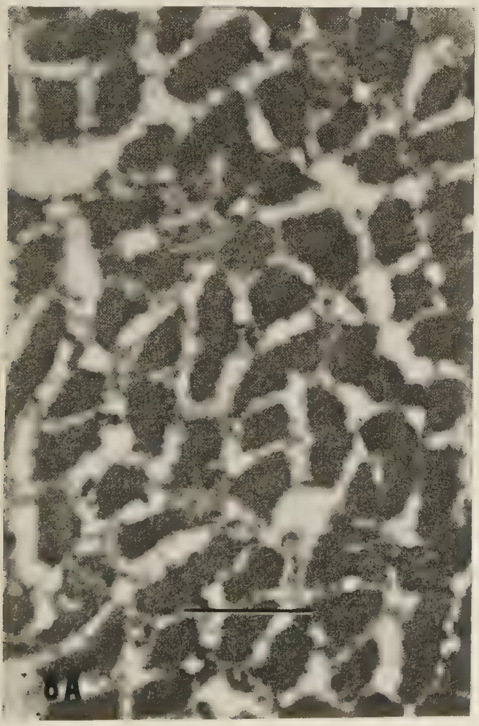
The lines on the photographs indicate 1 μ.

7 Extraocular muscles, Dalton fixed, embedded in methacrylate, electron micrographs, transverse sections of thin muscle fibers with concentrations of mitochondria (M) on their borders and located at the periphery of the extraocular muscles. A, Fibrillenstruktur; B, Felderstruktur.

8 Extraocular muscle fibers, fixed in glyoxal, teased, stained for cholinesterase, mounted in glycerine, nerve endings identified, refixed in Dalton, embedded in methacrylate, electron micrographs. A, from bundle of muscle fibers with "en plaque" endings only. All the muscle fibers appear this way. Fibrillenstruktur. B, from a bundle of muscle fibers, mostly with "en grappe" endings, but with a few "en plaque" endings. Most of the muscle fibers appear this way. Felderstruktur.


M
7A


M
7B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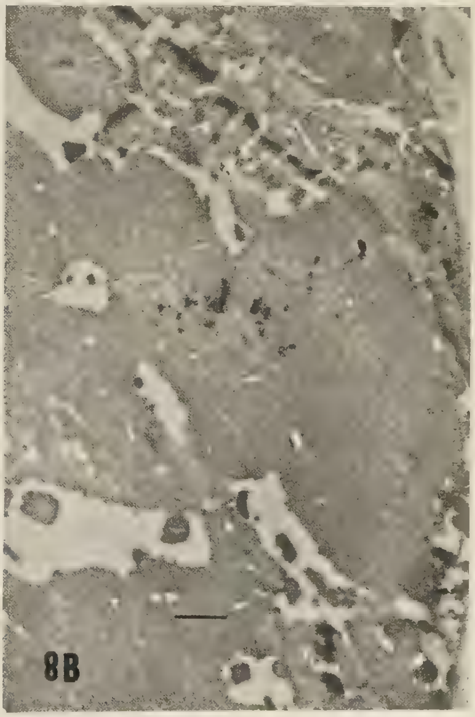

8B

PLATE 5

EXPLANATION OF FIGURES

Extraocular muscles, fixed in glyoxal, teased, stained for cholinesterase, mounted in glycerine. The lines on figures 9A and 10 indicate 10 μ. Figures 9A, 11, 13B, and 14 are the same magnification; figures 9B, 10, 12, and 13A are the same magnification.

9A and B Same muscle fiber. Multiple endings appearing as irregularly spaced spots of cholinesterase. Thin muscle fiber. It is suggested that this is the thin muscle fiber with concentrations of mitochondria at its borders located on the periphery of the extraocular muscles.

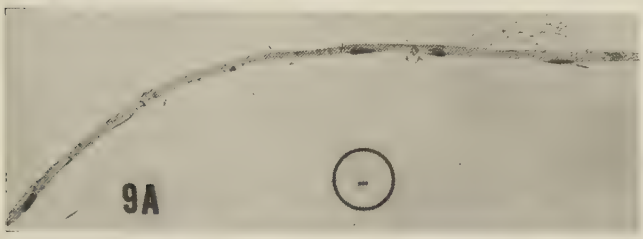
10 Multiple endings of simple form appearing as irregularly spaced spots of cholinesterase.

11 and 12 "En plaque" endings.

13A and B Same muscle fiber at different magnifications with "en grappe" endings.

14 Low power view showing mostly "en grappe" endings. "En plaque" endings are at the arrows.

9B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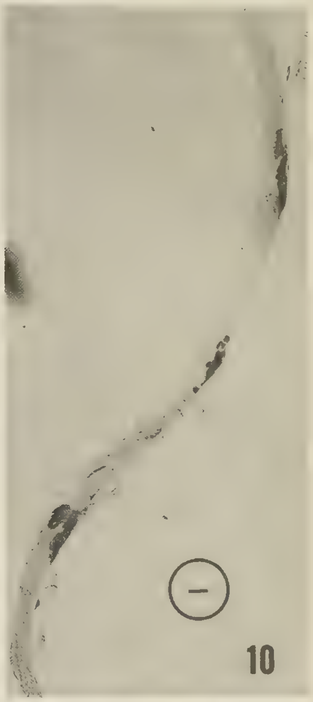
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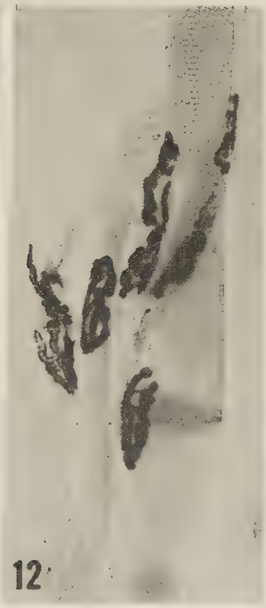
11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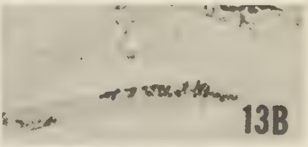
13A


13B

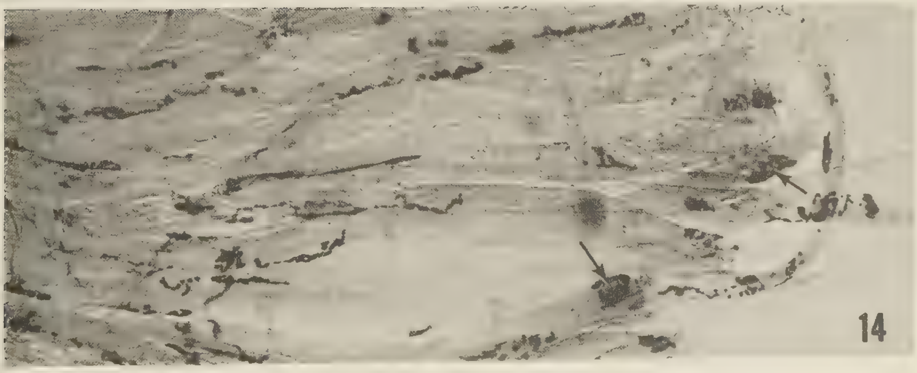

14

PLATE 6

EXPLANATION OF FIGURES

Extraocular muscles, fixed in glyoxal, teased, stained for cholinesterase, and mounted in glycerine. All figures are the same magnification. The line on figure 15 indicates 10 μ.

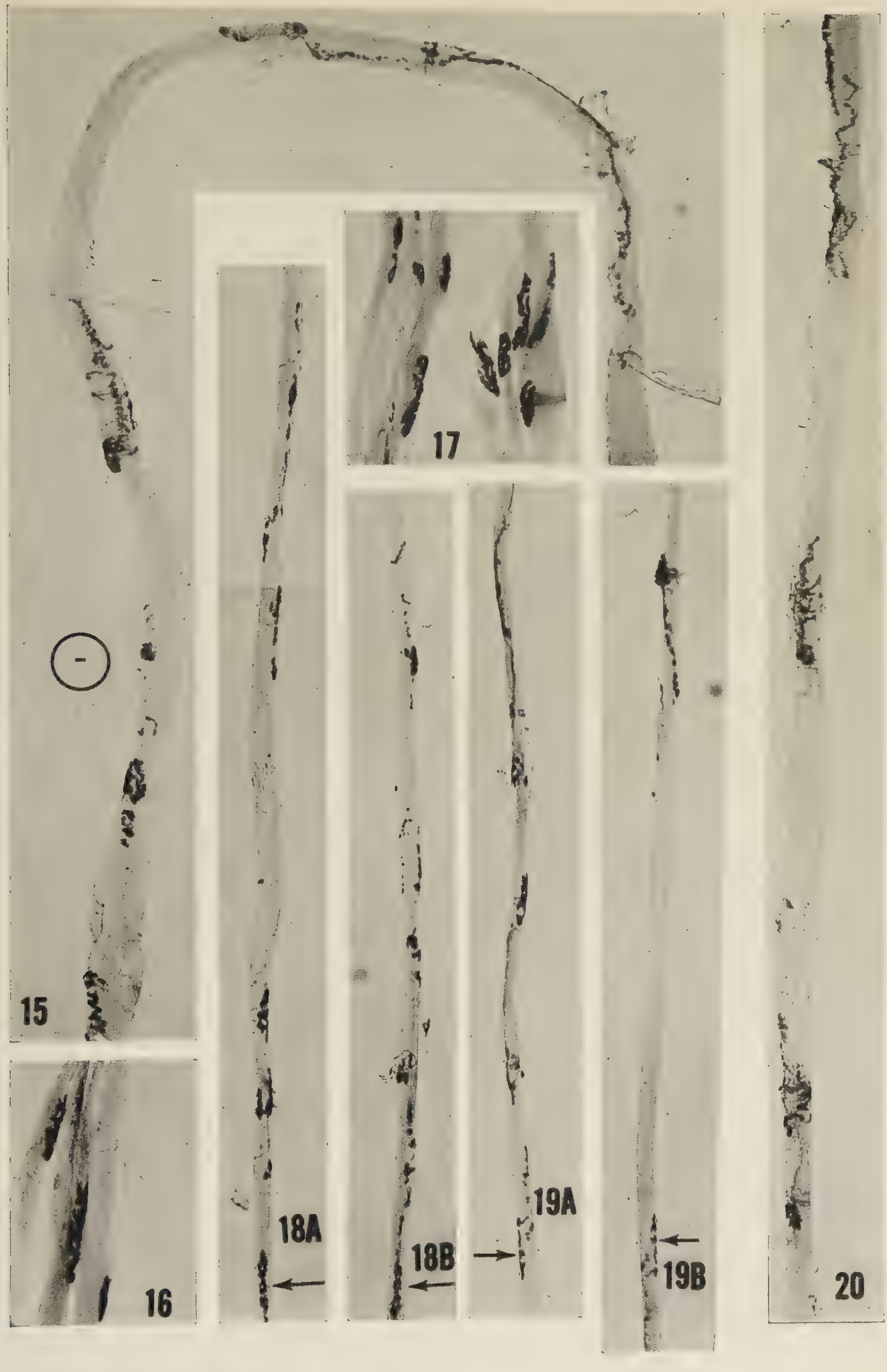
15 Montage. Multiple "en grappe" endings on a single muscle fiber.

16 and 17 "En plaque" endings at the same magnification as the other figures and included to show the differences in appearance between "en plaque" endings and the "en grappe" endings of the other figures.

18A and B Same single muscle fiber continuous at the arrows showing multiple "en grappe" endings.

19A and B Same single muscle fiber continuous at the arrows showing multiple "en grappe" endings.

20 Montage of single muscle fiber showing multiple "en grappe" endings.


15
16
17
18A
18B
19A
19B
20

Quantitative Measurements of Changes in Mouse Skin Following a Single Dose of Ultraviolet Light

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A single dose of ultraviolet light produces hyperplasia and other changes in the skin of the mouse ear that may persist for many days but ultimately subside. These changes have been described semiquantitatively (Blum et al., '59) together with a method of quantitative dosage. Repeated dosage of the same tissue results in cancer (see Blum, '59); the relationship, if any, between these two types of hyperplastic growth remains to be established.

In the present experiments attempt has been made to obtain quantitative, analyzable data which might throw light on the transitory hyperplasia that follows single doses of ultraviolet light; they have been exploratory in the sense that there seemed no way of knowing at the start, just what measurements might be useful. The plan followed was to make several kinds of measurements on the same material, that is, on the same histological sections. In this way relationships were revealed that had not been anticipated, and all the data were comparable and available ultimately for analysis. Analysis of epidermal hyperplasia in terms of a growing cell population, using these data, will be the subject of the following paper.

METHODS AND MATERIAL

Irradiation. The method of irradiating mouse ears has already been described (Blum et al., '59). The ears are held under a quartz plate normal to the beam of radiation. In the earlier studies the source of radiation was an intermediate pressure mercury arc, which was again used in the present studies; and, in addition, a Westinghouse Sterilamp (W 721-20) in a special glass envelope, which emits a high proportion of its radiation in the 0.2537 μ line. For our purposes, the latter may be regarded as a monochromatic source since wavelengths longer than 0.32 μ are ineffective. Both sources were monitored by means of a photocell and integration counter following the method used in this laboratory in a variety of studies (see Blum, Grady and Kirby-Smith, '42; Blum et al., '57). The system was calibrated in terms of energies incident at the plane of the ear (ergs cm^{-2}). Calibration of the low pressure mercury arc with a meter designed by Latarjet, Morenne and Berger ('53) for use with this type of source, agreed within 10% with the calibration by means of our photocell and integration counter. In terms of absolute energies this is within the expected error in measurement of radiation of these wavelengths.

Animals. As in the previous studies the animals were strain A albino mice weighing 20 to 30 gm; those used in the present study were bred from a small stock in this laboratory. In a few experiments litter mates were used, as indicated below. The animals were fed on purina Fox chow and were bedded on soft paper as previously described. After irradiation they were kept in the dark to avoid photorecovery (see Rieck and Carlson, '55), but previous to irradiation were subjected to alternate darkness and illumination, in contrast to the earlier studies where continuous darkness was maintained.

Histological method. The histological method was essentially that used in the earlier studies (Blum et al., '59), sections being cut at 10 μ thickness. As in those studies one-half of each ear was exposed to the radiation, the other half being shielded by a thin opaque screen placed between the quartz plate and the ear. For marking the position of this screen, the following technique was used: The edge of the screen was lined up over the median artery which is nearly straight for some distance after it appears at the base of the ear. When the ear was removed for histological treatment, a line was drawn on the outer surface with India ink following this median artery at the base and extending beyond as a straight line to the tip of the ear. The sections were cut perpendicular to this line and the fragment of the line retained in the section marks the position of the edge of the shield.

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Princeton University.

[2] National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland.

Measurements. All observations and measurements of the section were made using an oil immersion lens, distances being determined with a calibrated ocular micrometer. The most efficient method found was to obtain and record the desired information from each microscopic field of the tissue section as this was obtained. Use of a multiple Veeder counter facilitated recording. Six different morphological aspects were measured: (1) The number of intact epidermal cells per unit area of ear, determined by counting the total numbers of nuclei of epidermal cells along 100 μ of ear length in each field at all levels of focus. Since the sections were cut 10 μ in thickness the count corresponds to the number of cells in that volume of epidermis lying above 1000 μ^2 of dermis. All observable normal nuclei were counted regardless of their position in the epidermis, including pyknotic nuclei but not including those abnormal nuclei not in mitosis but lacking an intact nuclear membrane. (2) The incidence of mitotic figures and the phase of division were recorded for prophase, metaphase and anaphase-telophase, the latter included in a single category. Details of the method of counting are given in the next section. (3) The mitotic figures seemed randomly distributed in the normal epidermis, but after irradiation tended to be grouped together. The number of groups was recorded, when one or more figures were found close together they were counted as a group. (4) The mean diameter of the nuclei was determined for each field by averaging the long and short diameters of 5 nuclei. (5) The thickness of the epidermis excluding the corneum. This is the least accurate of the measurements since one lacks an accurate means of determining the outer limit. (6) The thickness of the dermis, measured as the distance from the layer of cartilage to the epidermis.

A procedure was established to minimize subjective error, which may enter particularly in the counting of mitotic figures. More than 100 sections of 10 μ thickness could be obtained from each ear and those sections chosen for study were at least 40 μ distant from one another. The number of sections studied for each experiment varied from 12 to 21, representing 300 to 400 fields. To avoid subjective error, 5 or 10 slides were counted at a time. The slides were labeled according to a code and then mixed; after each section was counted they were shuffled and one selected at random for counting the next section. The results of all previous counts were hidden from the view of the observer. The number of fields measured was determined by that required for accurate counting of mitotic figures (see below), this being the most difficult measurement and most subject to error. For greater uniformity in comparison, certain areas were avoided in choosing the sections to be counted; (1) the margins, where the anatomy is somewhat different from the rest of the ear, (2) the immediate region of the edge of the opaque shield, (3) the immediate neighborhood of open lesions (see Blum et al., '59).

Mitotic figures. The counting of mitotic figures is the most difficult of these measurements. Decision in identifying the mitotic figure introduces uncertainty, particularly with regard to prophase which is the most difficult to identify with certainty since the first changes that take place during cell division are subtle and likely to be missed. Since prophase is the longest phase of mitosis its incidence exceeds all others and its inclusion in the counts may be particularly important in detecting grouping of mitotic figures. In this study nuclei in prophase were identified as those in which the nuclear chromatin strands had become visible. In the previous studies three different observers counted the nitotic frequency in some of the same issue. They obtained different values, alhough these follow similar curves when lotted with respect to the time after iradiation (Blum et al., '59); and it would ppear that the criteria for judging the nitotic figures was different although self onsistent for each observer. Following he procedure outlined below for avoidance f subjective error, it was found that one bserver could, after rigorous training, repeat counts on the same material with nly 10% variation; all counts recorded here were made by this observer (G.A.S.).

To insure adequate sampling several fields were counted for each experiment, and the standard deviation calculated. From the variation of the S.D. it was estimated that for minimum mitotic frequency in the normal epidermis, at least 12,000 cells must be counted in order to obtain values which varied less than one standard deviation unit from the mean in 90% of the counts.

In these studies the proliferation rate is greatly increased after irradiation (see Blum and Soffen, '61) which means that the time of the total mitotic cycle decreases. In figure 1, the numbers of the different phases of the mitotic figure are compared without regard to the rate of proliferation, except that the highest points on the graph represent the greatest incidence of mitotic figures and hence the greatest rate of proliferation and shortest over-all mitotic interval. If the period oc-

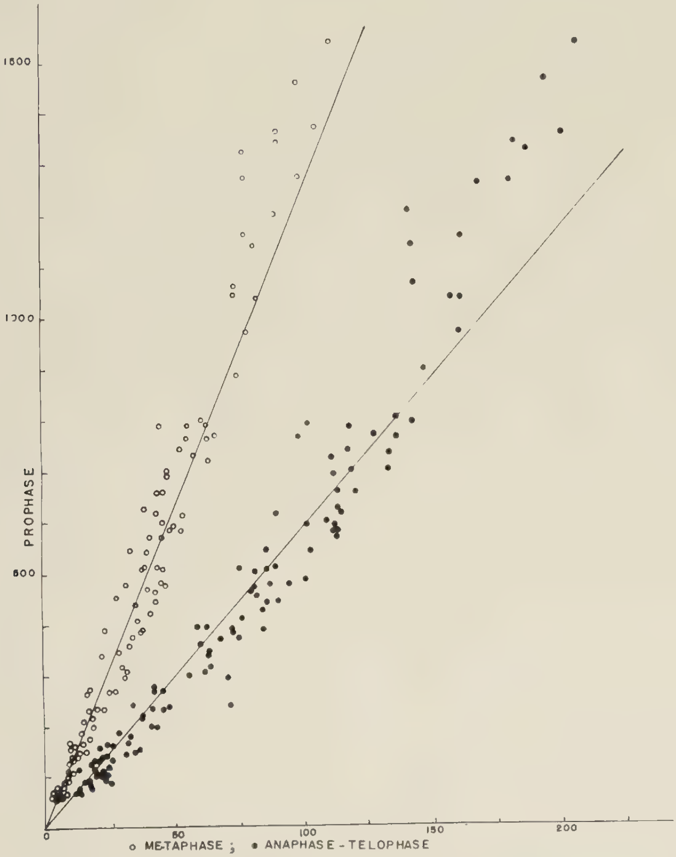

Fig. 1 Proportionality of phases of the mitotic figure. Each point compares the number of mitotic figures in anaphase-telophase, or in metaphase, with the number in prophase; for a given dose and time after irradiation. There is considerable scatter; and a systematic drift is indicated.

TABLE 1

Individual variation in incidence of mitotic figures

	Littermates (12 weeks old)	Non-littermates	Age
	mitotic figures per 1000 μ^2	*mitotic figures per 1000 μ^2*	*weeks*
	0.226	0.191	8
	0.215	0.234	9
	0.223	0.224	11
	0.201	0.204	12
	0.193	0.210	13
Mean	0.212	0.213	
S. D.	0.014	0.017	

TABLE 2

Reproducibility of incidence of mitotic figures

Exp.	Dose	Days after irrad.	Mitotic figures per 1000 μ^2			Per cent deviation
			Animal a	Animal b	Av.	
	erg cm^{-2} × 10^5					
		Low pressure mercury arc				
1	7.125	5.5	1.4198	1.1772	1.2985	9.4
2	14.250	2.5	0.4123	0.3481	0.3752	9.8
3	14.250	4.5	1.1930	1.0066	1.0998	8.5
		Intermediate pressure mercury arc				
4	12.0	5.5	0.9775	1.1643	1.0709	8.7
5	24.0	4.5	1.8852	2.3092	2.0972	10.3

cupied by the mitotic figure remained constant, or if the duration of the phases changed proportionally, the points in this figure should fall along a straight line. This does not appear to be the case, a systematic shift being evident. We may infer from the apparent deviation from linearity that the relative durations of the different phases of the mitotic figure, and hence the duration of the figure as a whole, decrease with increase in rate of cell division. This introduces an uncertainty into the use of the incidence of mitotic figures as a quantitative measure of the rate of cell proliferation, as will be pointed out in the following paper (Blum and Soffen, '61).

To indicate the amount of individual variation in unirradiated epidermis, mitotic incidences for 5 male siblings are compared in table 1 with those of 5 males from different litters and ages ranging from 8 to 16 weeks. The standard deviations are respectively 6.6 and 8.0% of the mean. The advantage in the use of litter mates did not seem to justify the difficulties incurred, and most of the experiments were carried out with mice within the range of ages indicated above.

To determine the reproducibility of mitotic counts in irradiated epidermis, experiments were carried out on 5 pairs of animals in which the treatment of both members of the pair was identical, but the dosage and time of counting were different for each pair. The results, listed in table 2, indicate good reproducibility. The internal consistency of the data represented in figures 3 to 7, also indicates the general accuracy of the counts (see also Blum and Soffen, '61).

Possible perturbing factors. Several factors that might affect the results of these experiments were explored.

As reported in the earlier paper (Blum et al., '59), minute open lesions appear on the ears following high dosage with ultraviolet light; they are much less frequent with the low pressure arc. It was thought that these lesions might be caused by the animals scratching the irritated area with their paws. To examine this possibility three animals were immobilized for 5 days after exposure of the ears to a high dose of ultraviolet radiation, by firmly binding the extremities. The number of open lesions that appeared was not detectably changed by this procedure; and as seen from table 3, the incidence of mitotic figures in regions not in the immediate neighborhood of the lesions was close to that for animals not restricted by binding. The lesions do not appear to be caused by scratching.

TABLE 3

Effects of scratching on incidence of mitotic figures

(Irradiation with low pressure arc 14.3 × 10^5 ergs cm^{-2})

	Bound	Unbound
	mitotic figures per 1000 μ^2	
	1.6524	1.7719
	1.8202	
	1.7048	
Mean	1.7255	

To determine the effect of pressure from the quartz slide, the ears of two mice were placed under the slide without being irradiated. The mitotic figure incidences were found to lie within the range of variation for normal epidermis in ears not subjected to this operation.

In these experiments the posterior half of the ear was usually the area exposed to the ultraviolet light, but in a few the anterior half was irradiated. The mitotic counts were consistent for the two areas, indicating that if the margins are excluded, as was routinely the case, there is no important variation in the effect of the radiation over the surface of the ear.

In the previous experiments (Blum et al., '59) all the animals were kept in total darkness both before and after irradiation; whereas in the present study they were kept on a 12-hour light, 12-hour dark cycle prior to irradiation, but in darkness for three days immediately afterward. The consistency of the earlier studies with the present ones indicate that normal illumination had no significant effect on the measurements as made here, either through photorecovery or control of normal mitotic rythm.

Diurnal rythms of mitotic activity in epidermis have been reported, and a longer cycle of hair growth and of mitotic activity also observed (see Montagna, '56). Ears were used in the present experiments without regard to such cycles, the consistency of measurements of incidence of mitotic figures indicating that these phenomena had no important affect.

Qualitative changes in the ear

Both gross and microscopic changes following irradiation with the intermediate pressure mercury arc, have been previously described (Blum et al., '59). Irradiation with the low pressure mercury arc produces the same qualitative changes, the only differences being of a quantitative kind. This is illustrated in plate 1 which shows three cross-sections of mouse ears; a normal ear in the middle, with an ear exposed to low pressure mercury arc radiation above, and one exposed to the intermediate pressure arc below. The irradiated half of the ear is to the right in both the latter. It is to be noted that the thickening of the ear, which is largely due to thickening of the dermis, is greater, in general, in the case of irradiation with the intermediate pressure arc. This is probably due to the deeper penetration of the radiation from the latter, as will be discussed in the following paper (Blum and Soffen, '61).

The ear irradiated with the intermediate pressure arc also shows a small open lesion with exudate extending over the neighboring surface. Such lesions, which were described previously (Blum et al., '59), appear much more frequently with the intermediate pressure arc, suggesting that they are initiated by changes in the deeper tissues rather than in the epidermis— that they are not due to scratching has been indicated above. As in the previous studies, the neighborhood of these lesions was avoided in making mitotic counts and other measurements.

Normally epidermal cells undergo degenerative changes, and are transformed into the flake-like elements of the corneum. The dead cells are replaced by proliferation in the malpighian layer. The proportion of cells being changed into corneum, and the corresponding prolifera-

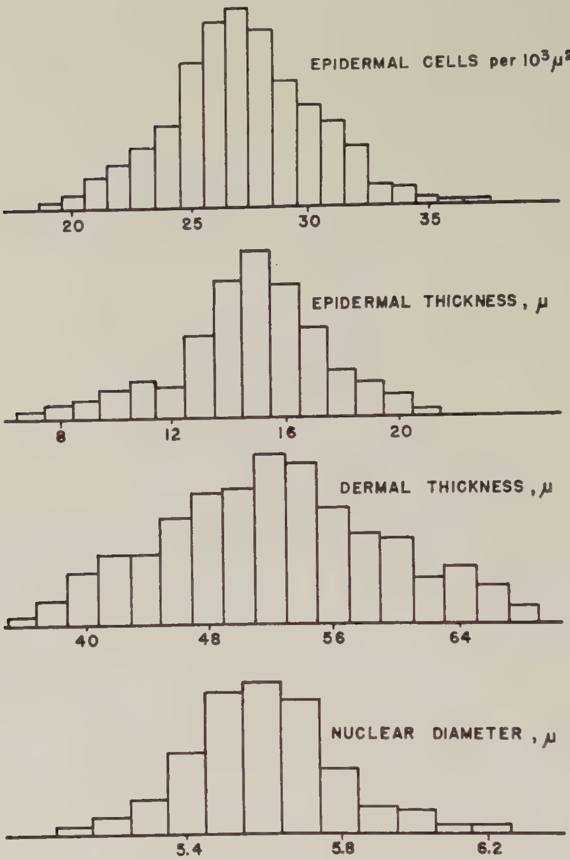

Fig. 2 Histogram showing distribution of aspects of the normal epithelium.

tion are relatively low in the normal tissue; but after irradiation this increases. Thus one sees increased numbers of mitotic figures, as will be discussed below, and also, an increased number of cells showing degenerative changes. This is particularly apparent in the number of cells showing dark, parakeratotic granules that form a distinguishable layer just beneath the corneum.

Marked swelling of the cells, and of their nuclei is observed in the irradiated epidermis. This is illustrated in plate 2.

Quantitative changes in the epidermis

The normal ear

Measurements on the normal ear provide a necessary basis of comparison for studying the changes effected by ultraviolet light. Figure 2 shows histograms based on 368 measurements on 5 unirradiated mouse ears, all the aspects represented are nearly normally distributed. The average epidermal thickness is 15.1 μ; S.D. 2.6 μ; the small standard deviation indicates that the thickness is quite constant. The number of epidermal cells per 1000 μ^2 is 27.2; S.D. 3.1. The mean nuclear diameter is 5.6 μ; S.D. 0.2 μ. The mean dermal thickness is 52.3 μ; S.D. 2.1 μ. The mean number of mitotic figures in the epidermis of normal ears from 5 animals was 78.5 per 10,000 cells. This corresponds to 0.21 mitotic cells per 1000 μ^2. For each of the 5 ears this value was close to that for the grouped data. These animals were not litter mates, their ages ranging from 8 to 16 weeks. It appears that this is a quite constant value.

Irradiated ears

Figures 3 to 7 describe the course of changes in 5 parameters measured during the 6 weeks following irradiation: figure 3, the incidence of mitotic figures per 10,000 epidermal cells; figure 4, the number of epidermal cells per 1000 μ^2; figure 5, the thickness of the epidermis; figure 6, the thickness of the dermis; and figure 7, the mean nuclear diameter. In each figure are plotted above and below, respectively, the data for irradiation with the intermedi-

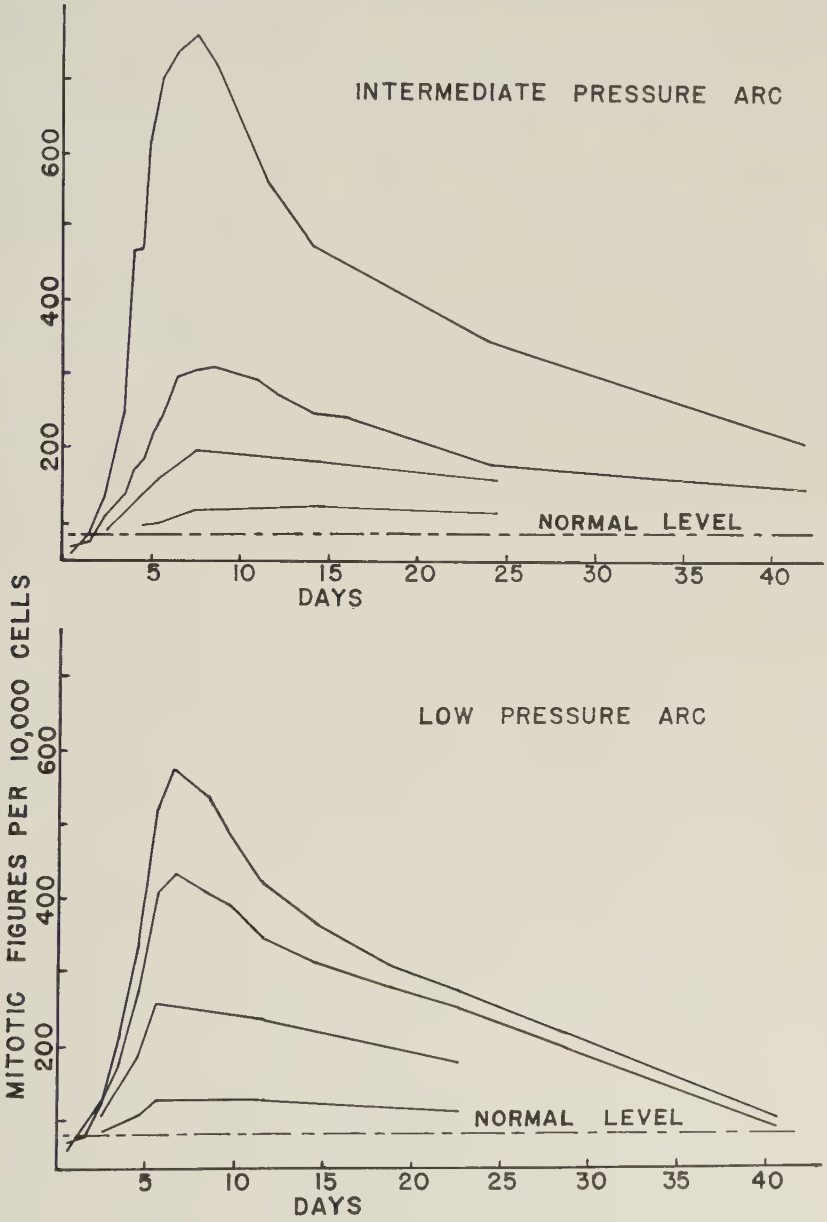

Fig. 3 Incidence of mitotic figures per 10,000 epidermal cells after irradiation with various doses of ultraviolet light. Doses in ergs × 10^5 cm^{-2} in order from top to bottom: Intermediate pressure arc 4.2, 8.4, 12.0, 24.4. Low pressure arc 2.4, 4.8, 7.1, 14.3.

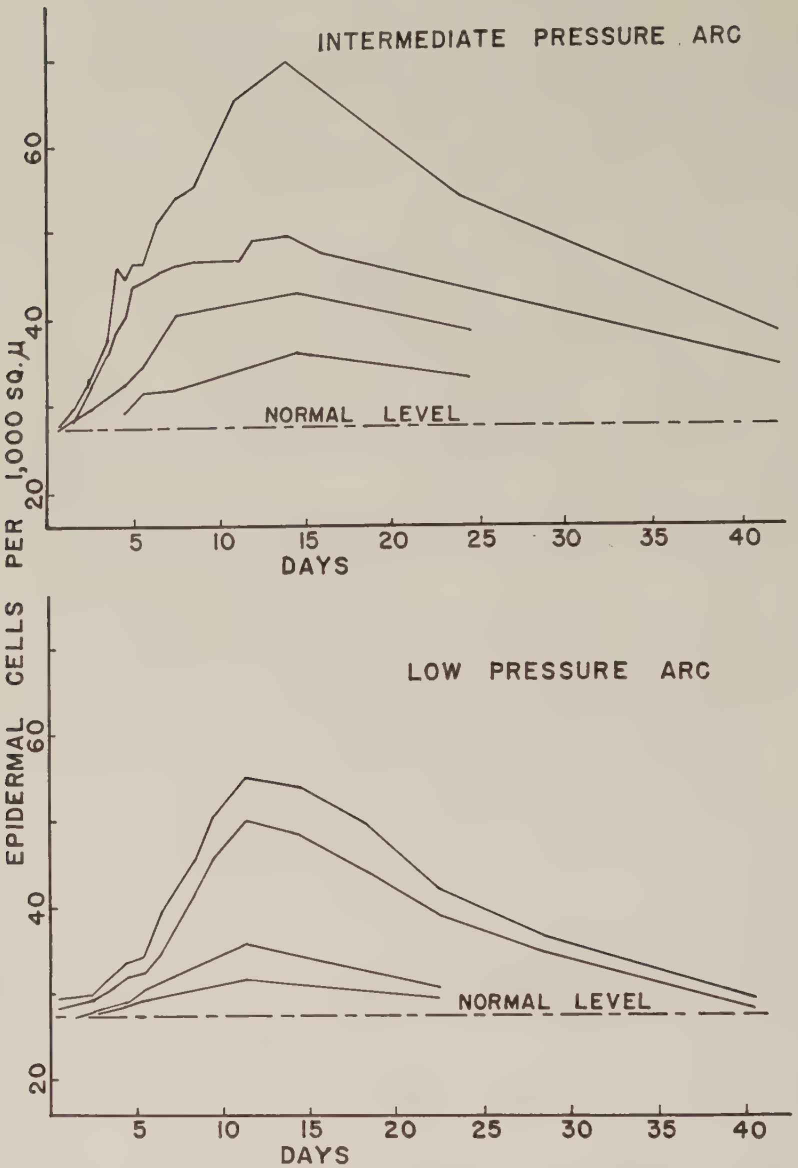

Fig. 4 Number of epidermal cells per unit area, after irradiation with various doses of ultraviolet light. Doses in ergs × 10^5 cm^{-2} in order from top to bottom: Intermediate pressure arc 4.2, 8.4, 12.0, 24.4. Low pressure arc 2.4, 4.8, 7.1, 14.3.

ate and low pressure arcs. In figures 3 to 7 only those doses are represented for which enough points have been obtained to describe curves rather completely. Limited measurements were made with other doses to study dose relationships, and these are used in the following paper.[3] A striking similarity in the course of these hanges is seen in all cases. With either ource of radiation there is first a relatively apid increase in the particular measurenent, which appears directly related to he dose. A maximum is reached, which s again related directly to the dose, folowed by a fall that is more gradual than he corresponding rise.

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[3] The original data are listed in the Doctoral thesis of G. A. Soffen. A quantitative study of the effects of single doses of ultraviolet light on mouse skin, Princeton University, 1960. These should be corrected as follows: Table 7: Low pressure arc, 9.5×10^5 ergs cm^{-2}, 22.5 days; value for epidermal cells per 10^3 μ^2 should read 41.8. Table 8: Intermediate pressure arc, dosage 20.4×10^5 ergs cm^{-2}, 24.5 days; value for mitotic figures per 10^4 cells, should read 325.1.

All these changes, with the exception of nuclear diameter, may be related to an increase in number of cells, that is, to a hyperplastic process, and it is of interest to note that the *hypertrophy* of the nuclei, which is also observed qualitatively in the cells themselves, follows the same general course. The measured nuclear diameter

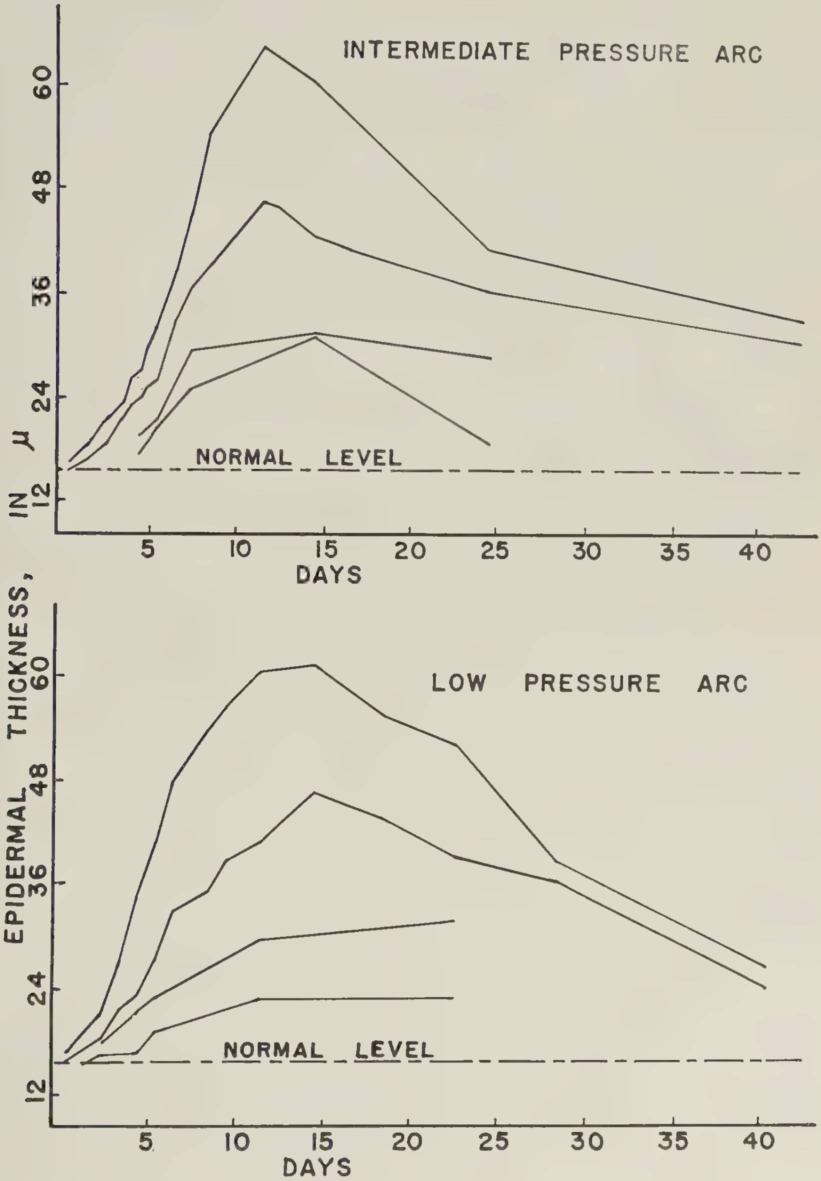

Fig. 5 Thickness of epidermis, after irradiation with various doses of ultraviolet light. Doses in ergs × 10^5 cm^{-2} in order from top to bottom: Intermediate pressure arc 4.2, 8.4, 12.0, 24.4. Low pressure arc 2.4, 4.8, 7.1, 14.3.

may increase as much as three times that of the normal cells. No doubt many of the smaller cells have been injured to the point that they cannot proliferate; we have no way of knowing to what extent the actively mitotic cells have been injured. The thickening of the epidermis obviously involves both hyperplasia and hypertrophy, and this is probably also true of the dermal thickening although it would be difficult to measure this. Some of the thickening is no doubt due to increased intercellular fluid (see Blum et al., '59).

As a measure of hyperplasia, that is, cell proliferation, the number of cells per unit area is a more useful index than the

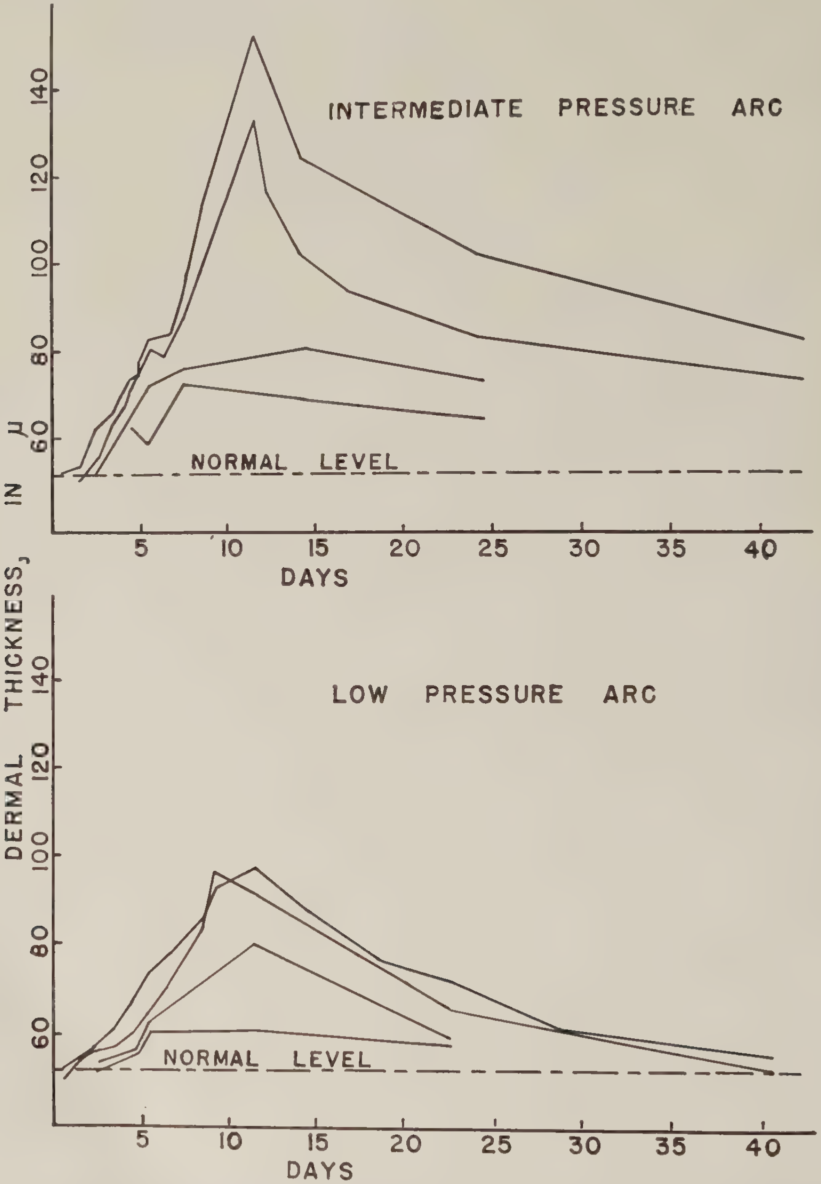

Fig. 6 Thickness of dermis, after various doses of ultraviolet light. Doses in ergs × 10^5 cm^{-2} in order from top to bottom: Intermediate pressure arc 4.2, 8.4, 12.0, 24.4. Low pressure arc 2.4, 4.8, 7.1, 14.3.

thickness of the epidermis, since swelling of the cells and intercellular edema are disregarded. And since the epidermis can grow only in one dimension, the number of cells per unit area also gives an index of the increase in cell population, when compared to the value for normal epidermis. That is, the number of cells per unit area before irradiation represents a population of cells in the normal state. After irradiation, this population increases by division of some of its cells, and, by following the increase of cells per unit area, we may expect to follow the growth of comparable

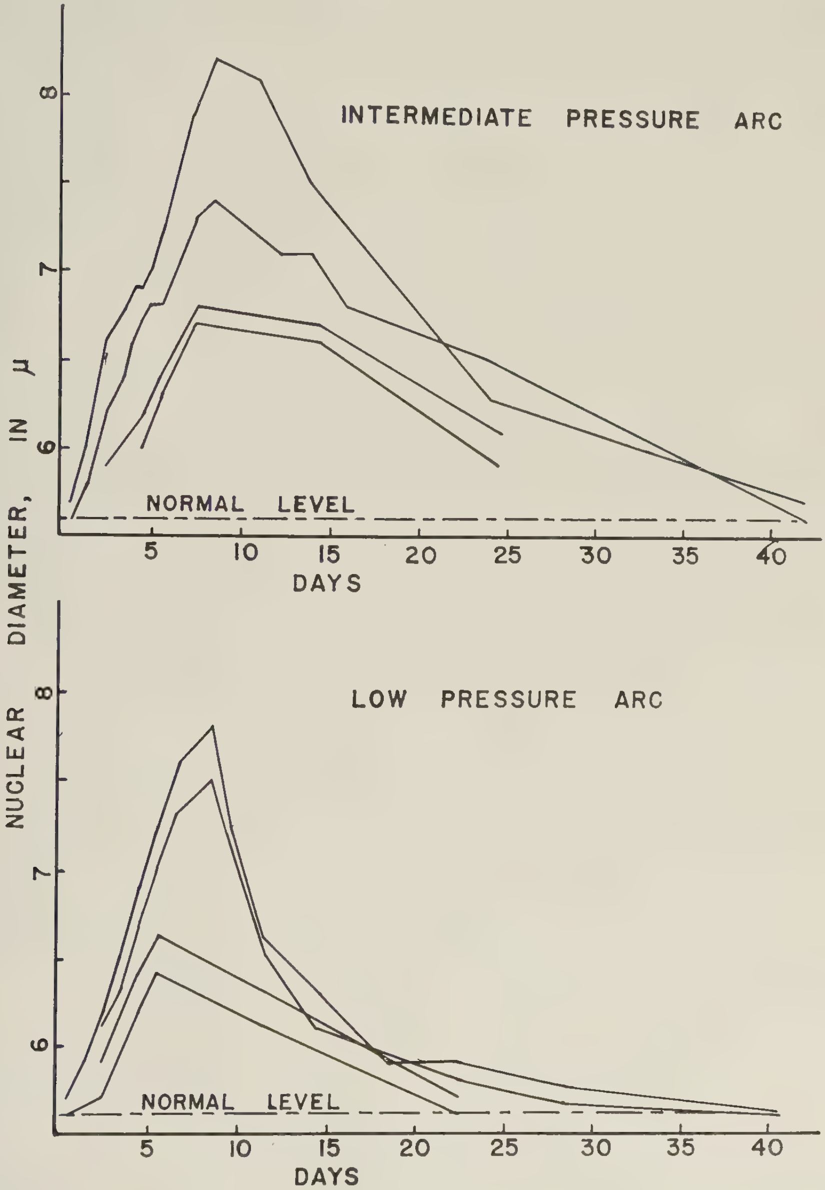

Fig. 7 Mean diameter of nuclei in epidermis, after various doses of ultraviolet light. Doses in ergs × 10^5 cm^{-2} in order from top to bottom: Intermediate pressure arc 4.2, 8.4, 12.0, 24.4. Low pressure arc 2.4, 4.8, 7.1, 14.3.

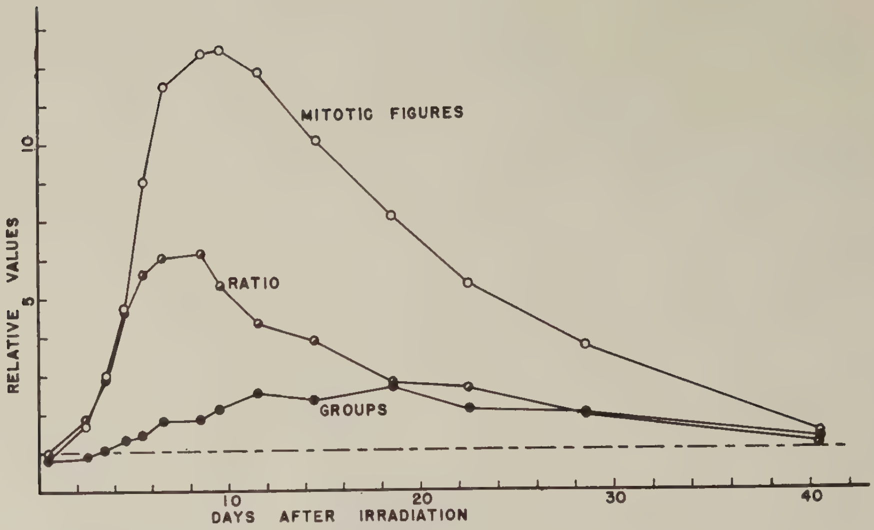

Fig. 8 Numbers of groups of mitotic figures after irradiation with intermediate arc; dose, 14.3 × 10^5 ergs cm^{-2}.

cell populations. Thus, if we wish to follow the rate of mitosis within a given population we should study the incidence of mitotic figures per unit area, rather than the commonly employed "mitotic index" used in figure 3. As will be seen in the following paper (Blum and Soffen, '61), the change in rate of mitosis may be analyzed to give us some insight into the process of hyperplasia. The values for incidence of mitotic figures per unit area used in figures 1, 2 and 3 of that paper were obtained by multiplying the frequency per 10,000 cells by the number of cells per mm^2. Obviously this measurement could have been made directly, and it might prove feasible to reduce the amount of labor in this way. But when the measurements were made, the study was in an exploratory stage, and it was not clear how they were to be used. This may have been fortunate, since it is to be noted that the method actually used eliminates any error that might arise from unequal shrinkage of the different tissue samples in the course of histological preparation. Plotting the data, corrected in this way, on a semi-logarithmic grid as is done in figure 1 of the following paper (Blum and Soffen, '61) yields good straight lines for a considerable part of both the ascending and descending arms of each of the curves.

In the unirradiated epidermis the mitotic figures are found in isolated cells distributed apparently at random. After irradiation, however, they tend to be grouped, as many as 7 having been found within close radius. In figure 8 is plotted against the time after irradiation: the total number of mitotic figures; the number of groups of mitotic figures; and the ratio of these numbers. To facilitate comparison the numbers of figures and the numbers of groups have been divided by the value for normal epidermis, so that all the curves begin at unity at zero time. It is seen that, whereas the total number of figures increases over 12 times, the total number of groups rises to less than three times the normal. The ratio goes as high as 6, indicating a great increase in the proportion of grouped figures after the irradiation. Figure 8, represents only one dosage, results for other doses are comparable.

What does the grouping of mitotic figures mean? The division of a cell results in two cells which may be thought of as exact duplicates born at the same time. If these cells enter into mitosis at about the same time, there should be a good chance of finding a group of two mitotic figures at the next division. If the cells continued to divide simultaneously at reg lar intervals we might expect to find increasing numbers of cells in the group as ime went on. Since it would seem unlikely hat all the cells would continue to divide in close synchrony, however, we would not expect to find the size of groups increasing indefinitely. But if there were in the whole population a relatively small number of dividing cells, we might expect to find these in groups rather than randomly distributed.

Briefly, then, the presence of the mitotic figures in groups rather than scattered at random, would seem to indicate that at a given time relatively few clones of cells were proliferating. In the unirradiated tissue only a few cells are proliferating at rather long intervals and since we find only isolated mitotic figures it seems that there is not great synchrony of division of daughter cells. After irradiation the rate of proliferation increases and at the same time the number of groups. It would seem likely that the more rapidly proliferating cells tend to divide more nearly in synchrony. This could account for finding only single cells in normal tissue since the chances of finding two cells in the same thin tissue section (10 μ) is very small if the group is no larger than this.

A simple calculation will show that only a relatively small number of cells can be proliferating, even in the most active tissue. Since each division produces two cells

$$N = N_0 2^d, \qquad (1)$$

where N_0 is the initial number of cells in the clone, and N the number after a given number of divisions, d.

Taking logarithms and rearranging

$$d = \frac{\log N - \log N_0}{\log 2} \qquad (2)$$

Let us take as an example data on irradiation with 24.4 × 10^5 ergs per cm^2 from the intermediate pressure arc, which represent the maximum observed rate of increase in incidence of mitotic figures. Plotting on a semi-logarithmic grid (see Blum and Soffen, '61) it is found that between 1.5 days and 5.5 days the incidence of mitotic figures increases at a constant relative rate. During this 4 day interval the incidence of mitotic figures rises from 237.7 per mm^2, which is within normal limits, to 3236.8 per mm^2. Inserting the latter figures for N_0 and N respectively in equation (2) we find that 3.8 divisions have taken place during 4 days, or a division every 1.05 days, that is, approximately once a day.

The calculation in table 4 is based on the assumption that the whole epidermal cell population is dividing once per day starting at 1.5 days. The calculated values mount much more rapidly than the observed. Assuming a cell volume of 10^{-6} mm^3 one sees that after 4 divisions there should be a layer of cells almost half a millimeter in thickness!

If, on the other hand, it is assumed that the number of proliferating clones corresponds to the number of mitotic figures in normal tissue a quite different result is obtained, as is seen in table 5. Here the number of cells calculated represents the doubling once per day of a cell population corresponding to the number of cells showing mitotic figures in normal epidermis. It is seen that these numbers agree well with the corresponding numbers of mitotic figures observed.

These calculations are based on the assumption that the number of mitotic figures is an accurate measure of the rate of cell proliferation, and in the following paper it will be shown that this assumption may not be strictly valid. Nevertheless, there can be little doubt that only a relatively small number of cells are dividing in the irradiated epidermis. Whether

TABLE 4

Calculations of growth of irradiated epidermis based on proliferation of all cells once per day

Days after irradiation	No. of cell divisions	Number of cells per mm²		Calc./Obs.
		Calculated	Observed	
1.5	–	2.96 × 10^4	2.96 × 10^4	1.0
2.5	1	5.92 × 10^4	3.18 × 10^4	1.9
3.5	2	11.82 × 10^4	3.74 × 10^4	3.2
4.5	3	23.68 × 10^4	4.45 × 10^4	5.3
5.5	4	47.36 × 10^4	4.63 × 10^4	10.2

TABLE 5

Calculation of growth of irradiated cells, based on proliferation once per day of a number of clones equal to the number of cells in normal epidermis

Days after irradiation	No. of cell divisions	Number of mitotic figures per mm²		Calc./Obs.
		Calculated	Observed	
1.5	–	213.3	237.7	0.90
2.5	1	426.6	416.9	1.02
3.5	2	853.2	924.4	0.92
4.5	3	1706.4	2097.3	0.81
5.5	4	3412.8	3236.8	1.05

these represent members of the same clones that were proliferating prior to irradiation is not directly determinable.

SUMMARY

Quantitative measurements have been made of the following aspects of the effects of single doses of ultraviolet light on mouse skin: (1) incidence of mitotic figures; (2) number of epidermal cells per unit area; (3) epidermal thickness; (4) dermal thickness; (5) mean nuclear diameter; (6) number of groups of mitotic figures. The accuracy of the various measurements is discussed. All the measured aspects change quantitatively in similar fashion following the irradiation; increasing rapidly at first, reaching a maximum and then falling gradually toward normal. It is shown that only a relatively small part of the epidermal population can be proliferating, or the rates observed would lead to a much greater thickening than is witnessed.

ACKNOWLEDGMENTS

We are grateful to Professor Elmer G. Butler and Professor Roger Pinkham for criticisms and suggestions regarding this and the following paper.

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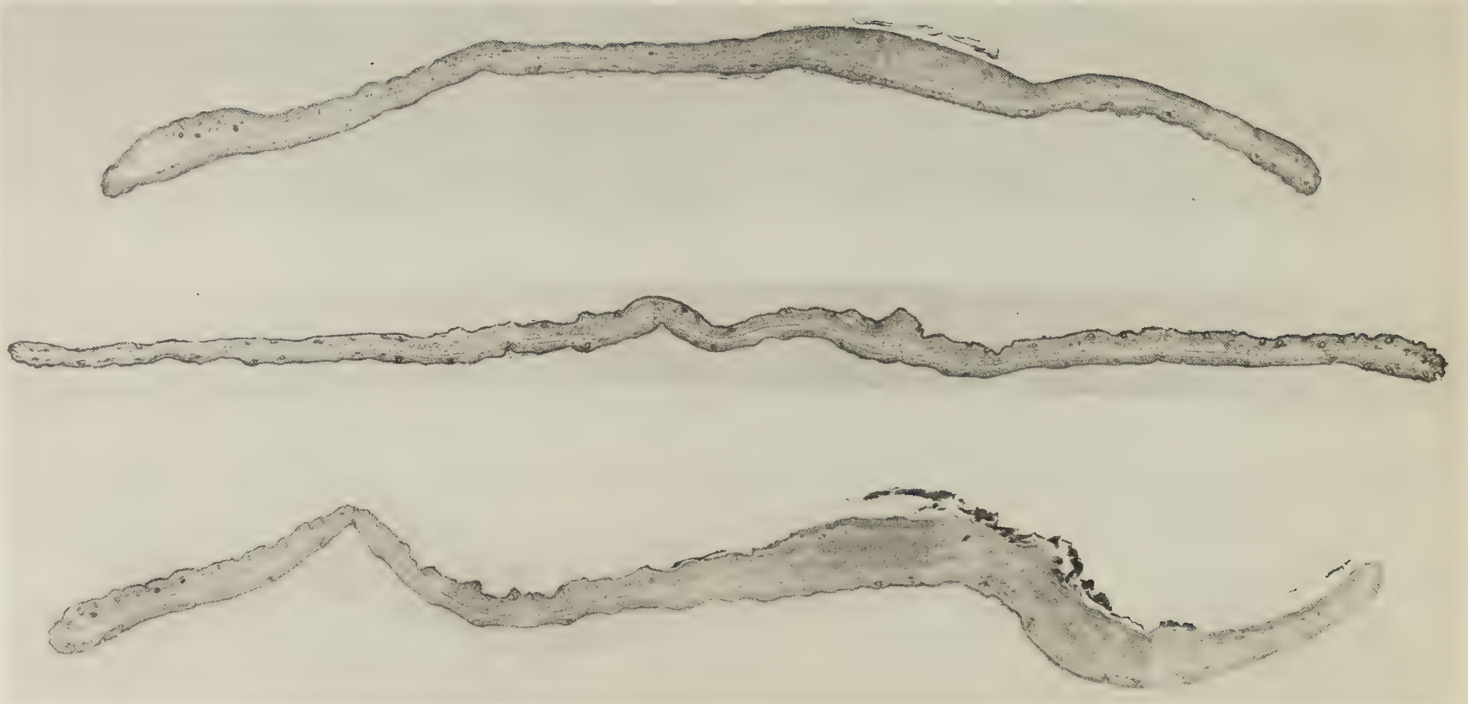
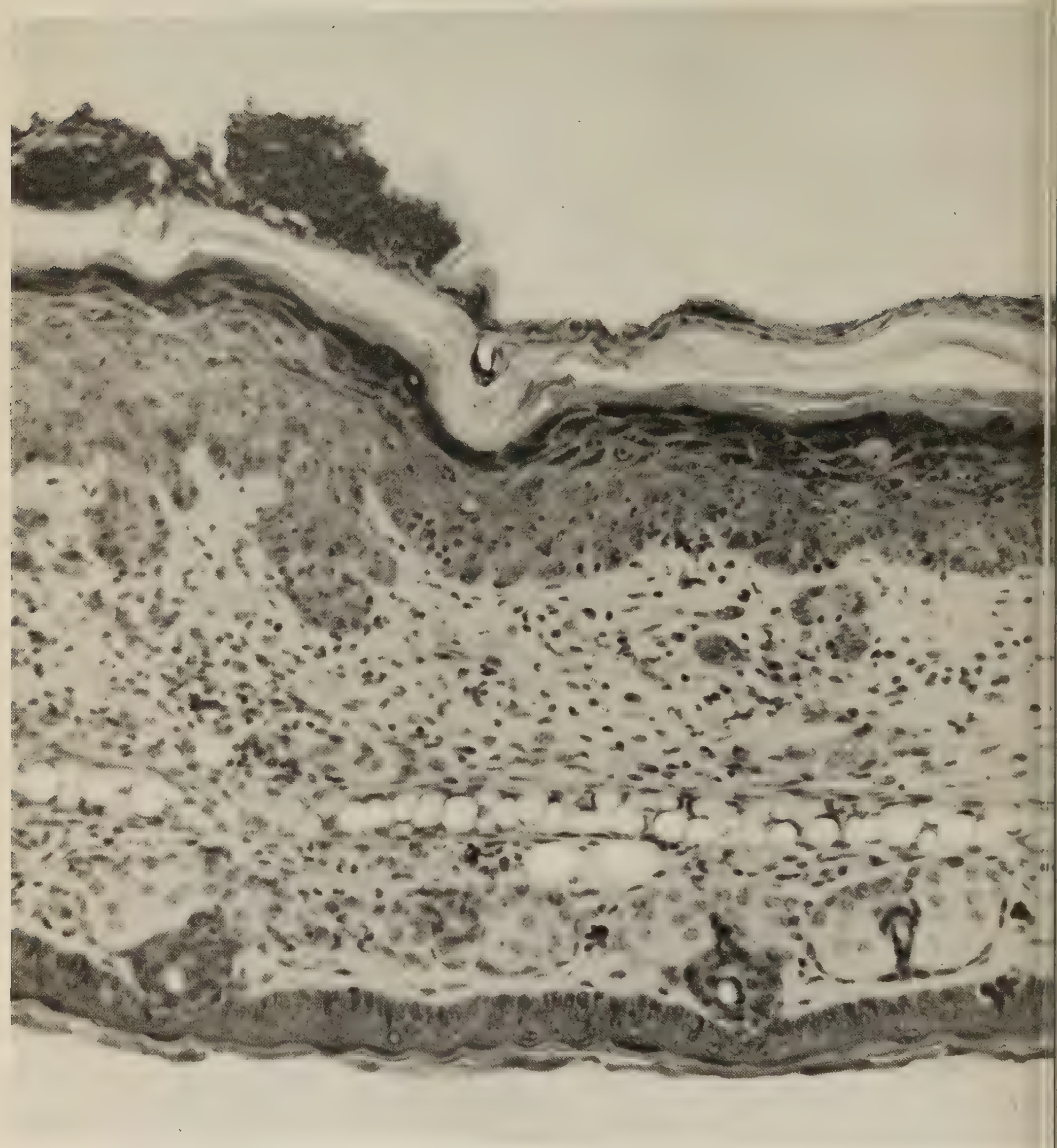
U-V LIGHT CHANGES IN MOUSE SKIN PLATE 1
Gerald A. Soffen and Harold F. Blum



Sections of mouse ears. Middle; normal ear. Top; right half irradiated 11.5 days earlier with low pressure mercury arc 14.2 ergs cm^{-2} 10^5. Bottom; right half irradiated 11.0 days earlier with intermediate pressure mercury arc 24.0 ergs cm^{-2} × 10^5.



Section of ear, irradiated 11.5 days earlier with low pressure mercury arc 14.2 ergs $cm^{-2} \times 10^5$. Note swollen cells and nuclei in epidermis of irradiated surface (top) compared with that of under surface which was not directly exposed.

Quantitative Analysis of Epidermal Hyperplasia in Mouse Skin Following Single Doses of Ultraviolet Light

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This paper deals with quantitative aspects of the hyperplasia that follows single doses of ultraviolet light, using the data described in the preceding one (Soffen and Blum, '61). The most plausible explanation seems to be that materials released by the cells which are injured by the ultraviolet, promote more rapid proliferation of the uninjured or only slightly injured fraction of the cell population. Although this explanation does not completely satisfy all quantitative aspects of the data, it seems possible to eliminate some alternatives. Whatever the ultimate explanation the present treatment should have at least heuristic value.

Cell proliferation

Since cell division always involves a doubling in number of some of the cells in a population, the growth of the population must at any moment be related to the number of cells in this dividing population. If the number of dividing cells is reasonably large, and they are not dividing in synchrony, the situation may be described as follows:

$$\frac{dN}{Ndt} = f(t) = \text{rate of proliferation} \qquad (1)$$

where N is the number of cells at time t, and f(t) is some function of t.

By integration

$$\ln N - \ln N_0 = \int_0^t f(t)dt = \text{growth by proliferation}, \qquad (2)$$

where N_0 is the initial number of cells, N the number of cells at time t, and ln the natural logarithm.

In reasoning quantitatively about hyperplasia it is of first importance to distinguish between rate and number of proliferating units since these two things may vary independently. The importance of this in the cancer problem has been discussed elsewhere by one of us (Blum, '59).

If the number of mitotic figures is proportional to the number of dividing cells we may write

$$\ln M - \ln M_0 = \int_0^t f(t)dt, \qquad (3)$$

where M_0 is the initial number of mitotic figures and M the number at time t. For purposes of analysis let us assume such proportionality for the time being; that is, write the equations in terms of numbers of cells, and apply the data on numbers of mitotic figures to these equations. Later on in the paper the uncertainty of the number of mitotic figures as a quantitative measure of proliferation will be discussed.

Equations (1) and (2) describe growth starting from a given initial population measured by N_0 and M_0, respectively. Subsequent measures, N and M, must include only the original population and its offspring. Since epidermis grows in only one dimension (increase in thickness) the population of cells or of mitotic figures above a given area should give an appropriate measure of N or M, respectively. Thus, the values of M used in the present treatment are given in terms of the number of mitotic figures per mm^2 of epidermis. These values are obtained, as explained in the preceding paper (Soffen and Blum, '61) by multiplying the number of mitotic figures counted per 10,000 epidermal cells, by the number of cells per mm^2. It is to be pointed out, that if the number of mitotic figures per cells counted (the usual "mitotic index") were employed, the relationships shown in figures 1, 2 and 3 would not have been obtained. The measure we have used should apply equally well if the whole population were proliferating, or only a part, as has been shown in the preceding paper (Soffen, '61) to be the case.

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[1] National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland.

If the cells divide at constant intervals, we say that proliferation goes on at a constant rate, that is f(t) is a constant. We may describe this condition by

$$\ln N - \ln N_0 = k\ t, \qquad (4)$$

where k is a constant. Plotting on a semi-logarithmic grid gives a straight line if this relationship is followed. This has been done in figure 1 where the logarithm of the number of mitotic figures is plotted against time. It is seen that the curves are comparable in form for the various doses supplied by both the intermediate and low pressure arcs.

In normal epidermis, where the number of mitotic figures remains constant, the decay of cells must take place just fast enough to compensate for new cells supplied by proliferation, since the epidermis does not thicken at an appreciable rate. But after irradiation there is a

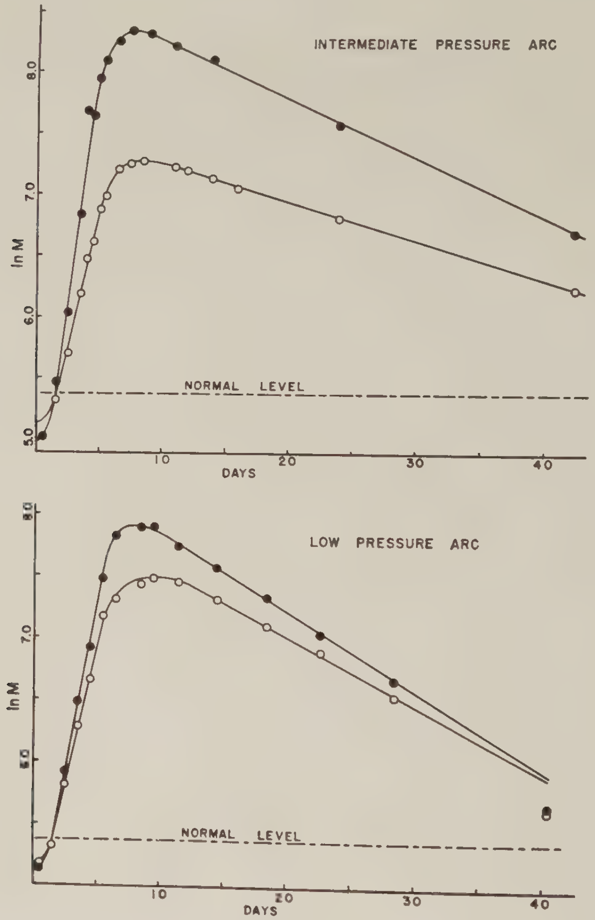

Fig. 1 Semi-logarithmic plotting of data on mitotic figure incidence from the preceding paper (Soffen and Blum, '61). The ordinate is the natural logarithm of the number of figures per mm^2 of epidermis (ln M). Only dosages are represented for which there is adequate data to describe the curve.

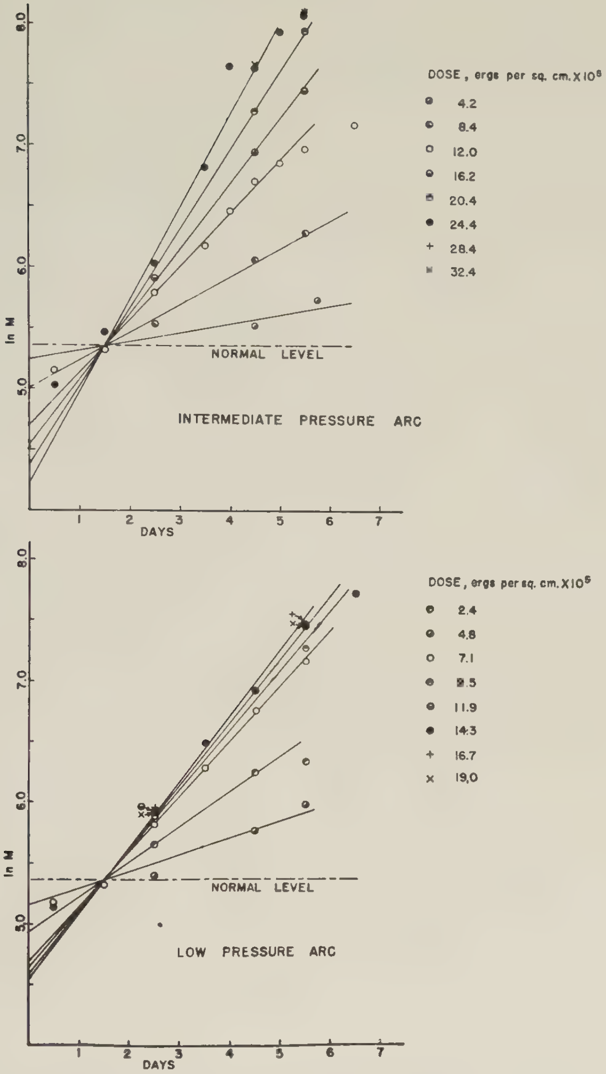

Fig. 2 Ascending, straight line portion of the mitotic figure incidence curves. Additional data are plotted which were not used in figure 1.

eriod when, as shown in figure 1, the urve follows a straight, ascending line; ndicating that proliferation is going on at constant rate. After a few days the slope f the curve diminishes, reaches a maxi-num and then falls off again, the descend-ng arm becoming a nearly straight line. his descending part of the curve needs ifferent explanation since there cannot e a negative rate of proliferation. The umber of epidermal cells falls off during nis period so it is clear that cells are decaying; but since the incidence of mitotic figures has been corrected for number of cells in a given population, decay of cells does not account for the falling off of this incidence. It seems clear that the rate of proliferation is falling during this time, as well as the size of the population. We have, then, to explain how after a dose of ultraviolet light, the rate of cell proliferation rises for a time at a constant rate and then eventually falls off, the rate of growth of the tissue becoming negative.

Another aspect that needs explaining may be pointed out. Focussing our attention on the early, ascending part of the curves, represented in figure 2, we see that the straight line portions intersect the horizontal line representing the level of mitotic figure incidence in the normal tissue, at nearly the same point, 1.5 days after the irradiation. This may give the impression that there is a "latent" period during which proliferation goes on at the normal level. But in such case it might be expected that the length of the latent period would vary with the dose rather than have a constant value as it appears to do. Another explanation presents itself. From the histological studies we know that dosage with ultraviolet light results in the ultimate death of some of the epidermal cells, as indicated by nuclear degeneration and parakeratosis. It seems reasonable, then, to assume that a good many of these cells are injured sufficiently to prevent their dividing, and that the population of dividing cells is for the moment reduced. Moreover, we see from figures 1 and 2, that the incidence of mitosis actually falls below that of normal tissue at 0.5 days, supporting the idea that the population of mitotic cells is reduced during an initial period, rather than that there is a true latent period during which nothing happens.

Surviving vs. dead cells

In order to explore possible mechanisms to explain these curves, let us begin with the initial photochemical process that sets off the physiological changes leading to the hyperplasia. The dose of ultraviolet light leads to the death of a certain fraction of the cells, the surviving fraction containing those which proliferate. We may assume that all the cells receive some radiation and are consequently injured to some extent, so that a sharp line probably cannot be drawn between dead or moribund cells and survivor cells. Nevertheless, it may be useful for purposes of analysis to draw this line, separating the cell population into "survivor cells" and "dead cells." Assuming that killing of the cells is first order we may write

$$N_s = N_n e^{-\gamma D}, \qquad (5)$$

where N_s is the number of cells surviving after the dose D, N_n is the initial number of cells in the normal tissue before the dose was given, and γ a constant.

The number of killed cells N_d is then represented by

$$N_d = N_n - N_s = N_n \ (1 - e^{-\gamma D}). \qquad (6)$$

The same form of equation would describe a first order photochemical reaction, in which N_n would represent the initial amount of some substance in the cells that was altered by the radiation, N_d representing the amount photochemically altered, and N_s the amount remaining unaltered.

A basic question to be asked in the present studies concerns whether the increased rate of proliferation of surviving cells results from the effect of the radiation on these cells themselves, or from the effect of the injured cells on the survivors, say be the release of substances from the former.

The proliferation curve

Let us first examine the latter possibility, assuming that a substance S, perhaps a product of the photochemical reaction, is gradually released into the tissue where some of it is used by the cells and some of it removed through circulatory channels. The rate of release of S should in this case be proportional to the amount remaining to be released, and we may write

$$S = S_0 e^{-\alpha t}, \qquad (7)$$

where S is the amount of the substance at time t, α is a constant and S_0 is the initial amount of substance at the moment of irradiation.

Let us assume that the rate of proliferation is proportional to S, but is also constantly decreased by a linear function which may represent the removal or using up of the substance in one way or another proportionately to the proliferation of the cells. Thus

$$\frac{dN}{N\,dt} = S - \beta, \qquad (8)$$

where β is a constant representing the linear function above mentioned.

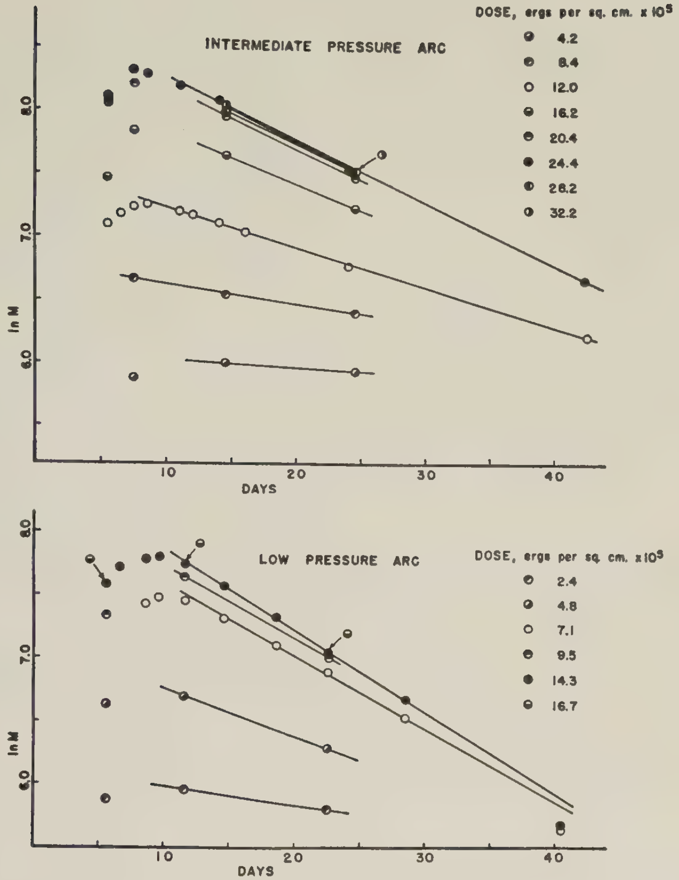

Fig. 3 Descending, straight line portion of the mitotic figure incidence curves. Additional data are plotted which were not used in figure 1.

Substituting from (7) in (8)

$$\frac{dN}{N\,dt} = S_0 e^{-\alpha t} - \beta. \qquad (9)$$

But S_0 should be proportional to the number of cells killed by the radiation and from (6)

$$S_o = \delta\ N_d = \delta\ N_n\ (1 - e^{-\gamma D}), \qquad (10)$$

where δ is a constant

Substituting in (9)

$$\frac{dN}{N\,dt} = \delta N_n\ (1 - e^{-\gamma D}) e^{-\alpha t} - \beta. \qquad (11)$$

Integrating between limits

$$\ln N - \ln N_s = \delta\ N_n \left[1 - e^{-\gamma D}\right]\left[\frac{1 - e^{-\alpha t}}{\alpha}\right] - \beta t. \qquad (12)$$

It is to be noted that while equation (12) indicates a rise and then a fall in *growth* of the population, equation (11) from which it comes, shows that the *rate* of proliferation is constantly falling.

For a given dosage the exponential containing D is, of course, constant, and for convenience in examining the shape of one of the curves let us collect constants as K, writing

$$K = \delta\ N_n(1 - e^{-\gamma D}). \qquad (13)$$

And substituting in (12)

$$\ln N - \ln N_s = K\left(\frac{1 - e^{-\alpha t}}{\alpha}\right) - \beta t. \qquad (14)$$

When αt is small,

$$(1 - e^{-\alpha t}) \cong \alpha t, \qquad (15)$$

to a close approximation, and substituting in (14) and simplifying

$$\ln N - \ln N_s = Kt - \beta t. \qquad (16)$$

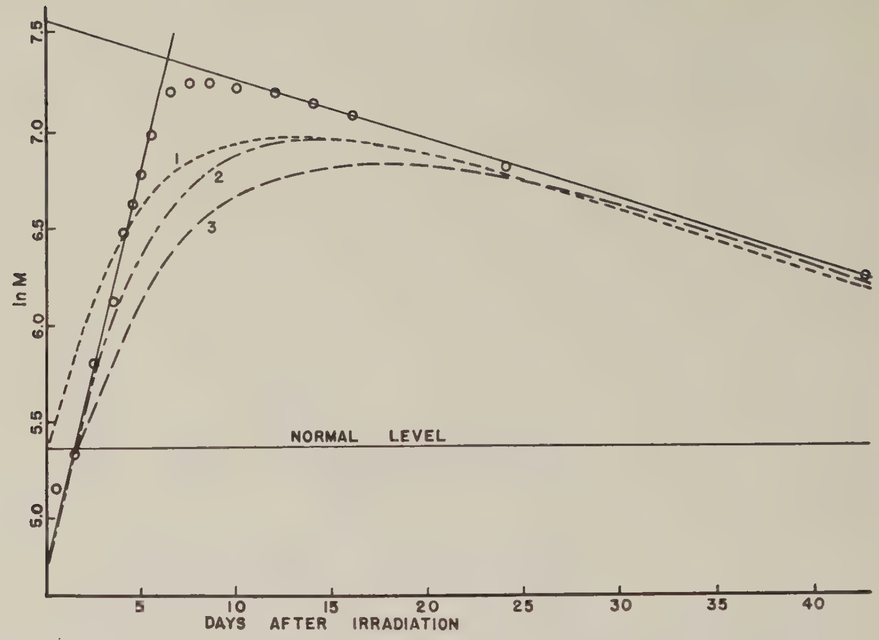

Fig. 4 Open disks represent the data on mitotic figures for intermediate arc dosage 12.0×10^5 ergs cm^{-2}. See text for explanation of the calculated curves 1, 2 and 3.

Thus, K being larger than β it would seem that during an early period the curve would be a straight line with positive slope, as observed in figure 2.

But the term $(1 - e^{-\alpha t})$ approaches unity as t increases so that for high values of αt, equation (14) would become, to a close approximation

$$\ln N - \ln N_s = K/\alpha - \beta t. \qquad (17)$$

The term K/α being constant, this could account for the straight line with negative slope that forms the later part of the curves, as seen in figures 1 and 3.

Applying equations (16) and (17) to the portions of the curve which they should represent, it is possible to obtain, graphically, values for the various constants which can be applied in (14). In (17), β is the slope of the descending part of the curve and K/α the intercept of the projected straight line on the ordinate. From (16) it is seen that K should be the sum of the ascending and descending slopes. Figure 4 shows curves calculated in this way for a dose for which the data are among the most complete. In making the calculations one may have a choice of origins; curve 3 starts from the normal level of incidence of mitotic figures whic the curve intercepts at t = 1.5 days; curv 1 starts from this level but at t = 0; curv 2 starts from the intercept on the ordinat corresponding to t = 0.

Clearly, none of the calculated curve in figure 4 accurately describes the dat although they are of the general form e pected. In all three cases the ascendin part of the calculated curve is not as near a straight line as is observed, nor is th slope as great at any point. Also, the max mum appears too late in the calculate curves. Substituting other constants, or i troducing slight modifications of equatio (14) do not improve the fit. But as will b shown, alternative hypotheses seem to e counter greater difficulties than the failu of our simple equation to give an exact to the data. At best this equation shou be regarded as an oversimplified approx mation that leaves out functions whic might account for the difference betwee calculated and observed curves. The u certainty as to where to take the startin point for the curves illustrates the impro ability that an exact fit is to be found wit out further information that is not fea ble to obtain. As will be shown below, the greatest discrepancy may reside in the inadequacy of the number of mitotic figures as a measure of cell proliferation.

Dose relationships

Before exploring alternative hypotheses and possible sources of deviation from the explanation just offered, it will be well to examine dose relationships in the same terms.

Let us assume, as above, that the early ascending straight line part of the curve is fitted by equation (16) and write,

$$\frac{\Delta \ln N}{\Delta t} = K - \beta. \qquad (18)$$

The right hand term represents the slope of the ascending straight line part of the curve, the last term, β, being the slope of the descending straight line part. From (18) we see that the sum of these two slopes gives a value for K. The ascending and descending slopes measured graphically from figures 2 and 3 are listed in table 1. Values of K obtained from these are plotted against D in figure 5, where we note that the curves for both the intermediate and low pressure arcs are nearly straight lines for low values of D, but level off rather sharply at high values.

Using the same kind of argument as in (15) to (17), we may write for low values of γD

$$(1 - e^{-\gamma D}) \cong \gamma D, \qquad (19)$$

and substituting in (13)

$$K = \delta\ N_n\ \gamma D. \qquad (20)$$

The slope

$$\frac{\Delta K}{\Delta D} = \delta N_n\ \gamma \qquad (21)$$

may be obtained graphically for each of the curves from the early straight line part, in figure 5. The values are 0.034 and 0.066, respectively, for the intermediate and low pressure arcs.

For high values of D, the term $(1 - e^{-\gamma D})$ approaches unity, and again substituting in (13)

$$K \cong \delta\ N_n \qquad (22)$$

This value can be estimated from the height of the curve at which it becomes virtually horizontal; the values obtained in this way are, respectively, 0.803 and 0.605 for the intermediate and low pressure arcs.

Combining (21) and (22) we thus obtain values for γ; 0.042 for the intermediate, 0.109 for the low pressure arc. Substitut-

TABLE 1

Dose	a ascending slope[1]	d descending slope[2]	K = a + d
ergs cm^{-2} × 10^5			
	Intermediate pressure		
4.2	0.072	0.007	0.079
8.4	0.228	0.015	0.242
12.0	0.422	0.035	0.457
16.2	0.535	0.041	0.576
20.4	0.647	0.049	0.696
24.4	0.751	0.050	0.801
28.2	0.751	0.053	0.804
32.4	0.751	0.055	0.806
	Low pressure arc		
2.4	0.137	0.015	0.152
4.8	0.290	0.028	0.318
7.1	0.457	0.058	0.515
9.5	0.492	0.058	0.550
11.9	0.522		
14.3	0.542	0.064	0.606
16.7	0.542	0.063	0.605
19.0	0.542		

[1] From figure 1.

[2] From figure 2.

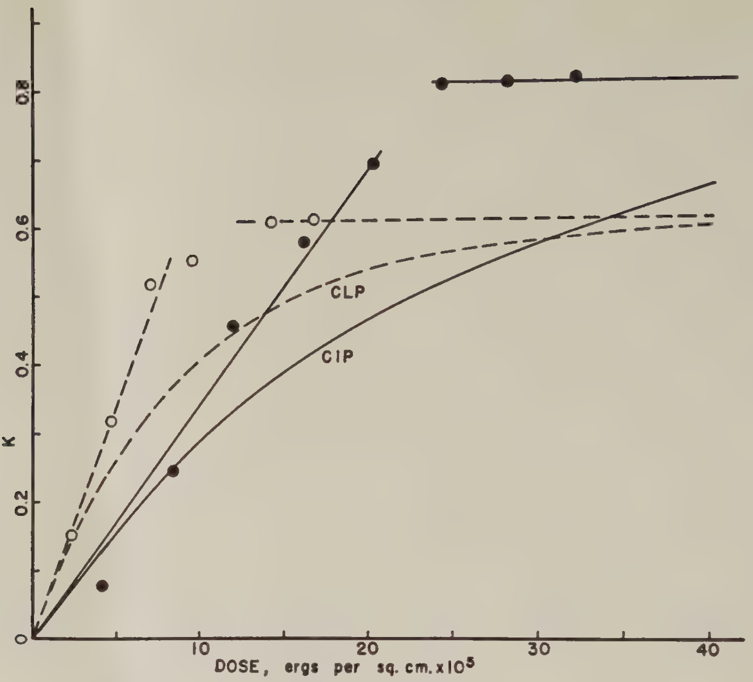

Fig. 5 Dosage relationships for epidermal proliferation. Intermediate pressure arc, solid disks and solid lines; low pressure arc, open circles and broken lines; CIP and CLP, calculated curves for these arcs, respectively. See text for explanation.

ing these values in (13) we calculate the curves CLP and CIP in figure 5. Clearly the fit is not good although the general shape of the curves is as predicted.

It may be pointed out that the relationship between the curves for the intermediate and low pressure arcs is that which might be expected. To explain this it is necessary to take into account the spectral emission of the two sources, and the spectral transmission of the skin tissue. In passing through the dead corneum the radiation is greatly attenuated by absorption enhanced by scattering in that layer. The radiation is selectively absorbed, longer wavelengths, e.g., the strong 0.2937 μ, 0.3020 μ, and 0.3130 μ lines of the intermediate pressure arc are absorbed to a much less extent than the 0.2537 μ line (Kirby-Smith, Blum, and Grady, '42). The latter wavelength, which constitutes practically all the effective radiation emitted by the low pressure arc, has thus been reduced much more by the time it reaches the living cells of the epidermis (the malpighian layer) than have the longer wavelengths. But, again, the shorter wavelengths being absorbed to a greater extent in the malpighian layer should have greater proportionate effect there; the longer wavelengths penetrate to a greater extent into the dermis where they are absorbed and they may have their principal locus of action in the thicker layer. This is a rather complex situation not easy to analyze quantitatively, but it is to be expected that the low pressure mercury arc would have greater effect in the epidermis relative to its effect on the dermis, than would the intermediate pressure arc.

The greater absorption of the radiation from the low pressure arc in the malpighian layer corresponds to a higher value of γ; hence to a greater slope for the early part of the curve in figure 5, and a lower maximum. Thus the general relationships between the curves for the two sources of radiation are those that would be expected. The relative effects on epidermis and dermis are indicated in figure 6. Here the

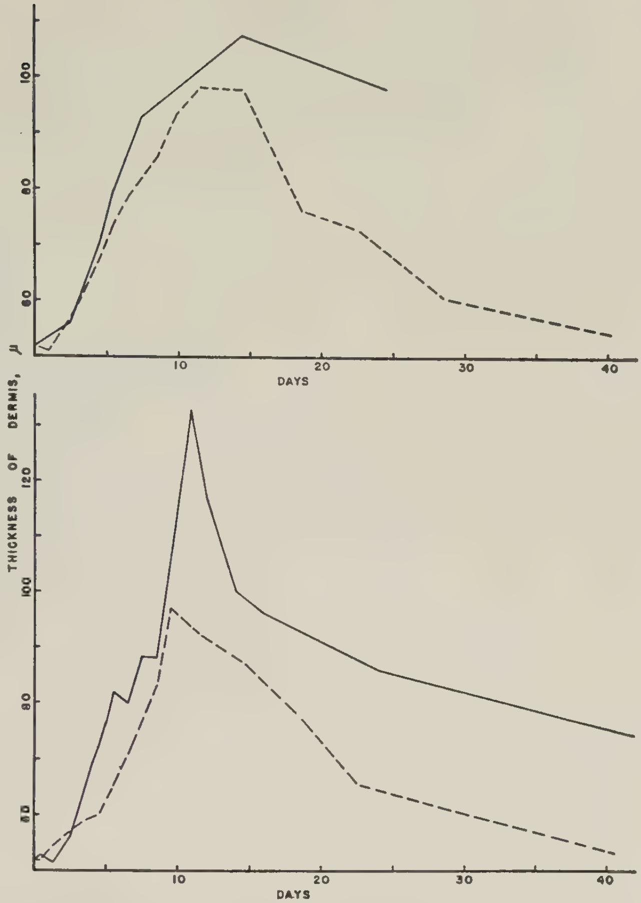

Fig. 6 Dermal thickening for comparable proliferation of epidermis. Solid lines; irradiation with intermediate pressure arc. Broken lines; irradiation with low pressure arc. Upper curves: Doses are 16.2 and 14.3 × 10^5 ergs/cm^{-2} for I. P. and L. P. arcs, respectively. Lower curves: Doses are 12.0 and 7.1 × 10^5 ergs/cm^{-2} for I. P. and L. P. arcs, respectively. References to table 1 will show that epidermal proliferation is comparable for these pairs of doses.

course of dermal thickening is compared for doses of intermediate pressure and low pressure arc radiation that produce comparable initial rates of epidermal proliferation, as indicated by comparable slopes in figure 2. It is seen that the dermal thickening is greater for the intermediate arc, as would be predicted.

Let us now examine the initial part of the curves where incidence of mitosis falls below that of the normal epidermis. The number of surviving cells should be reduced below the normal level according to the dose, as indicated in equation (5), and correspondingly the number of cells capable of proliferating. We have assumed above that the surviving cells proliferate at the same constant rate before the normal level is reached as they do afterward, and substituting from (5) in (12) we write

$$\ln N - \ln N_n e^{-\gamma D} = \delta N_n \left[1 - e^{-\gamma D}\right]\left[\frac{1 - e^{-\alpha t}}{\alpha}\right] - \beta t. \qquad (23)$$

The last term would be very small for the period of time in which we are interested (1.5 days) so let us neglect it, and substituting from (15), and (19) in 23, write for the period of time, t_n before the normal level is reached

$$\ln N_n - \ln N_n \ e^{-\gamma D} = \delta \ N_n \ \gamma \ Dt_n. \qquad (24)$$

Simplifying and rearranging

$$\ln N_n - \ln N_n = \delta \ N_n \ \gamma \ Dt_n - \gamma \ D = 0, \qquad (25)$$

and rearranging and cancelling out

$$t_n = \frac{1}{\delta N_n}. \qquad (26)$$

We note that D has been eliminated and that t_n is equal to a constant, hence all the curves should intercept at the normal level, as they do in figure 2. If one introduces the values of δN_n, obtained above into (26) the values of t_n obtained for the intermediate and low pressure arcs, respectively, are 1.25 and 1.65, which are not too far from the 1.5 observed.

We have indulged in considerable oversimplification in arriving at our conclusion and may even have "fudged" a little in drawing the curves in figure 2. But, although the data permit some leeway there would seem little indication of a systematic change in the position at which the curves intercept the horizontal line representing the normal incidence of mitotic figures, unless at very low dosage. The lines might have been drawn with slightly different slopes if they had not been brought to a common intersection at this point, but this would not have affected materially the dose relationship indicated in figure 5.

There is a certain unreality, of course, in the projection of the curves as straight lines below the normal level. It seems unlikely that proliferation would start off at a constant rate immediately after the dosage with ultraviolet light. There is ample evidence that this agent slows cell division, and that recovery toward the normal rate takes place rather slowly (e.g., Blum et al., '50, '56). More likely, then, the early part of the curves should be convex upward, and this is indicated by the position of the 4 values obtained for mitotic figure incidence at 0.5 days (see fig. 2). These values all fall below the normal level, thus indicating a reduced number of proliferating cells at this time rather than a "latent" period. Although it would be necessary to replace the constant in the above equations with some function of t other than a linear one in order to describe the early course of proliferation, it seems that the argument presented above could be essentially correct.

Validity of the measures and assumptions

The development of the above model was based on the data for numbers of mitotic figures, assuming these to furnish an accurate measure of cell proliferation. Examination of this assumption indicates that it is not strictly valid; and this may account for at least some of the discrepancies between the model and these data.

The chances of observing a mitotic figure should be proportional to the ratio of the duration of the figure to the time occupied by the whole cycle of cell division. Thus, the number of mitotic figures may serve as a measure of the relative number of dividing cells. This measure should be an accurate one under either of two conditions; (1) the duration of the mitotic figure is proportional to the duration of the whole cycle of division, or (2) the figure occupies only a negligible part of the whole cycle. It is generally assumed that when rate of cell division changes the interphase is affected more than the phases of the mitotic figure, and there is evidence for this in some cases (e.g., see Blum, Kautzmann and Chapman, '54). If the duration of the mitotic figure changed proportionally with the mitotic cycle as a whole, the different phases of the figure would also be expected to change proportionally; but figure 1 of the preceding paper (Soffen and Blum, '61) indicates that this is not the case. We are left with the second possibility, but this condition does not seem to be fulfilled either, as indicated by another discrepancy.

The difference in number of cells per unit area of irradiated and unirradiated epidermis might be taken as a measure of the number of cells added by proliferation; but since all the epidermal cells are not actively dividing (compare tables 4 and 5; Soffen and Blum, '61) we have no base line for comparison in relative terms. This difficulty is illustrated in figure 7 where the logarithm of the increase of cell number is plotted, for a given dosage, in two ways; in obtaining curve A, it was assumed that all the epidermal cells were proliferating (a maximum estimate); whereas in curve B the dividing population corresponds to the number of mitotic figures in unirradiated epidermis (a minimum estimate). The true course of proliferation should lie somewhere between these two curves. Clearly neither curve A or B could be directly compared with those in figure 1.

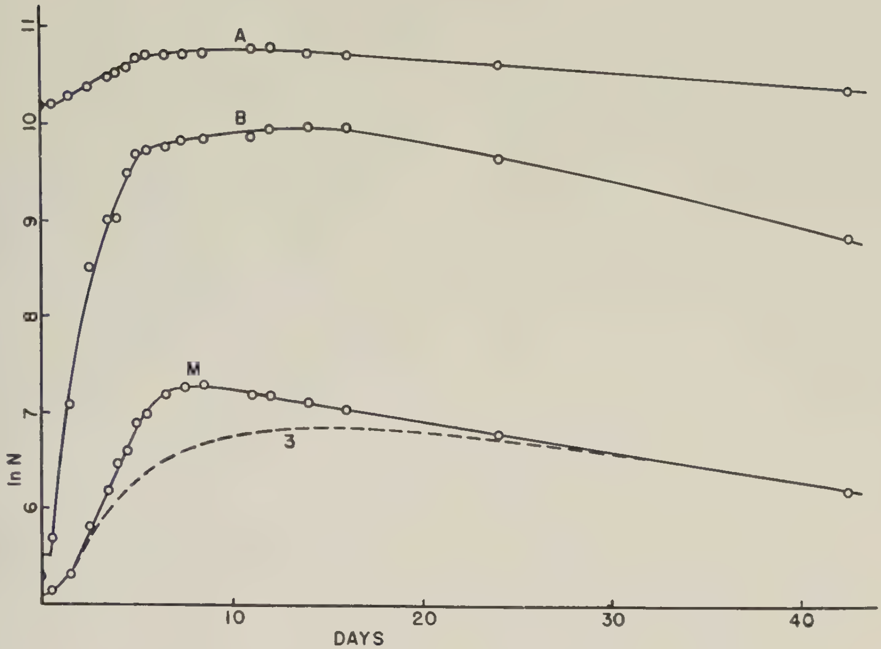

Fig. 7 Aspects of epidermal growth after dosage with 12.0×10^5 ergs/cm^{-2} of radiation from the intermediate pressure arc. A and B, logarithm of number of cells per cm^2: A, based on initial number equal to that in unirradiated epidermis; B, based on initial number equal to the number of mitotic figures in unirradiated epidermis. M, logarithm of number of mitotic figures per mm^2; 3, calculated curve from figure 4.

An important point is to be noted, however, in comparing figures 4 and 7, which represent the same set of results. The data for mitotic figures reach their maximum value much earlier than the curves based on number of cells, indicating a definite discrepancy between these two measures of cell proliferation. It seems unlikely that the kind of observations that are available can provide a reliable quantitative measure of proliferation. Improved techniques of counting either mitotic figures or population growth, would not eliminate such basic difficulties as are illustrated in figure 7, which would seem to enter into most, if not all, measures of proliferation in organized tissues.

The general shape of the curves in figure 7, seems to agree better with the calculated curves in figure 4, than with the data for mitotic figures on which the latter are based, particularly as regards the position of the maxima. This may be taken to support the model, although the equations cannot be directly applied to the data on numbers of cells.

There are other assumptions in the model that might be challenged; but with this major uncertainty they may not be worth laboring. Certain ones may be mentioned, however. The categorical separation of the population into dead and survivor cells immediately after the irradiation, which was arbitrarily assumed for purposes of analysis, can hardly pertain in an exact sense. A spectrum of degrees of injury is to be anticipated, and the behavior of the cells to vary correspondingly. For instance, it is not improbable that some of the injured cells may go through one or two cell divisions and then succumb—this is observed in bacteria, for example. The effect of such factors on the proliferation curves would seem difficult to predict. The assumption of a first order process for killing is also somewhat arbitrary. It may be mentioned that the rate of hemolysis of erythrocytes by ultraviolet light, which involves permeability changes, increases with the square of the dose (Cook, '56; Cook and Blum, '59).

Some alternative hypotheses explored

In spite of the uncertainty in interpreting the measurements, it may be profitable to consider some alternative hypotheses. In developing the above model it was assumed that the rate of proliferation is governed by some material released from the dead cells, which acts upon the survivor cells. Alternatively, we might have assumed that a substance is produced within the cells as a result of the radiation, and that this substance is gradually lost from these cells, say into the surrounding medium, the rate of release being proportional to the amount remaining. In this case the dead cells would play no role. The rate of proliferation would be related only to the amount of photochemical change within the individual survivor cells rather than to any such change in the cell population as a whole. In this case the same form of equations could apply, provided that material released did not affect the proliferation rate of other cells.

There is good qualitative evidence, however, that material released by the irradiated cells does cause increased proliferation. In an earlier paper (Blum et al., '59) it was shown that after irradiation, increased numbers of mitotic figures are found in epidermis not reached by the radiation but in the neighborhood of the irradiated area. It was also shown in the preceding paper (Soffen and Blum, '61) that the cells swell progressively after irradiation, following a time curve not unlike that for the incidence of mitotic figures and other aspects of the data directly related to hyperplasia. The swelling indicates increased permeability, favoring exchange of material with tissue fluids, and the time course suggests that the release may follow a curve not unlike that for mitotic figures. In figure 1 it is seen that the number of mitotic figures returns more slowly to normal after dosage with the intermediate pressure arc than after dosage with the low pressure arc. The former source has greater effect on the dermis (e.g., fig. 6) and hence would be expected to release a greater amount of proliferation inducing material into the tissue; this could account for the more gradual return toward normal in this case. All this evidence favors the concept that proliferation inducing material is released by the injured cells.

Since the idea of somatic mutation is often introduced into concepts of cancer let us consider it in the present regard. Imagine that the action of ultraviolet light on the epidermis is to produce mutations, or something akin thereto, in some of the cells, which cause them to proliferate more rapidly. By analogy with the inactivation of viruses, where a single quantum inactivates a virus particle, let us assume that a single quantum causes a mutation. The number of non-mutated cells would then be proportional to the number of surviving cells, and the number of mutants, N_m, should be represented by

$$N_m = k_1 N_n \ (1 - e^{-\gamma D}), \qquad (27)$$

where k_1 is a proportionality constant.

If the change in rate of proliferation were due to a mutation, it should be "all or none," and hence there should be no relationship between dose and rate *per se*. We might then write

$$\ln N - \ln N_n e^{-\gamma D} = k_1 \ (1 - e^{-\gamma D}) \ k_2 t, \qquad (28)$$

where k_2 is a rate constant for proliferation.

Simplifying, collecting constants as K_1 and rearranging we obtain

$$\ln N - \ln N_n = K_1 \ (1 - e^{-\gamma D}) \ t - \gamma \ D, \qquad (29)$$

which has a form similar to the equations used above to describe the early course of proliferation if a substance is released by the injured cells. The difficulty is that there seems no way to account for the descending part of the curve, which would be compatible with genetic or biophysical concepts of what constitutes a mutation. Moreover, such an explanation would not account for the qualitative evidence for release of material that promotes proliferation. The idea of a somatic mutation seems hardly applicable here—it also meets serious difficulties when applied to cancer induction by ultraviolet light (Blum, '59).

Let us examine the possibility that the rate of proliferation is a function of the number of survivor cells, the dead cells playing no role. Then we might write in place of (13)

$$K_2 = f_2 \ (N_n e^{-\gamma D}), \qquad (30)$$

where K_2 measures the rate of proliferation and is some function of the number of surviving cells, which is the quantity enclosed in parentheses. It is clear that the rate would in this case fall off with increase in dose, oppositely to what is observed.

It is sometimes postulated that in normal tissue the proliferation of cells is inhibited by the presence of a large population of normal cells; when cells are injured these presumably stimulate or release inhibition of proliferation of the normal cells until such time as a new normal population is formed. Besides introducing rather vague concepts with the terms "stimulation" and "inhibition," this hypothesis seems to meet quantitative difficulties. It would seem necessary to assume that the rate of proliferation would in this case be some function of the ratio of dead to injured cells, and we may write similarly to (30)

$$K_3 = f_3\left[\frac{N_n(1 - e^{-\gamma D})}{N_n e^{-\gamma D}}\right]. \qquad (31)$$

Canceling out and simplifying

$$K_3 = f_3(e^{\gamma D} - 1). \qquad (32)$$

We see that the rate of proliferation would be expected to rise with the dose, with no tendency to approach a maximum level as do the curves in figure 5.

DISCUSSION

Since no exact quantitative description of the data has been found, it seems inadvisable to assign a definite mechanism; but a resumé of alternative hypotheses seems in order.

The equations that give the closest approximation to the data permit more than one explanation: (1) the cells killed by the radiation release substances that increase the rate of proliferation of surviving cells; (2) a photochemically produced substance in the surviving cells which gradually decreases in amount, causes the increased proliferation of these cells; (3) a "somatic mutation" causes temporary increase in the rate of proliferation of the survivor cells.

The first hypothesis accounts best for the increase of proliferation in tissue which has not been directly irradiated.

It seems somewhat more difficult to account in terms of the second hypothesis for the evidence of release of materials that increase proliferation in the neighboring tissue.

In this case the released material would seem necessarily small in amount, whereas the observed effect on the unirradiated tissue is considerable (see Blum et al., '59).

The third hypothesis meets a seemingly overwhelming difficulty of explaining how the mutation can be "reversed" so that the proliferation rate falls off in the course of time. It also fails to account for the qualitative evidence of release of a proliferation accelerating factor into the tissues that have not received radiation.

The idea that a large population of living cells inhibits proliferation and that killing of cells thus leads to increase in proliferation seems ruled out on the basis of dosage relationships.

We seem faced in any case with the necessity of assuming that a material released into the tissue promotes increased proliferation, and we may inquire as to the nature of this material. One can only suggest that since it increases the rate of a process, it is something in the nature of a catalyst—an enzyme or possibly a coenzyme, which is temporarily increased in amount. Contrast may be drawn with the idea of the "tem" introduced by one of us to account for the "inherited" increase of proliferation in cancer tissue (Blum, '59).

SUMMARY

Analysis of aspects of the data on epidermal hyperplasia after single doses of ultraviolet light, indicates that neither number of mitotic figures, nor number of cells per unit area provide quantitative measures of cell proliferation.

A quantitative model has been constructed, which under the circumstances cannot be rigidly applied to the data; but which finds most tenable the hypothesis that the rate of cell proliferation of the surviving cells depends upon materials released from injured cells. Alternate hypotheses are considered.

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CONTENTS

R. E. GOSSELIN AND GAIL O'HARA. An Unsuspected Source of Error in Studies of Particle Transport by Lamellibranch Gill Cilia . . . 1

BRUCE M. EBERHART. Exogenous Enzymes of *Neurospora* Conidia and Mycelia . . . 11

R. E. GOSSELIN. The Cilioexcitatory Activity of Serotonin . . . 17

F. B. ADAMSTONE AND A. B. TAYLOR. Inhibition and Recovery of Golgi Response in Intestinal Epithelial Cells of the Rat Injected with Monoiodoacetic Acid-Treated Fat-Induced Chyme . . . 27

HERBERT SHAPIRO. Epinephrine and the Latency of Muscular Contraction . . . 35

HARVEY ASTERITA AND DOUGLAS MARSLAND. The Pellicle as a Factor in the Stabilization of Cellular Form and Integrity: Effects of Externally Applied Enzymes on the Resistance of *Blepharisma* and *Paramecium* to Pressure-Induced Cytolysis . . . 49

ARTHUR HESS. The Structure of Slow and Fast Extrafusal Muscle Fibers in the Extraocular Muscles and their Nerve Endings in Guinea Pigs . . . 63

GERALD A. SOFFEN AND HAROLD F. BLUM. Quantitative Measurements of Changes in Mouse Skin Following a Single Dose of Ultraviolet Light . . . 81

HAROLD F. BLUM AND GERALD A. SOFFEN. Quantitative Analysis of Epidermal Hyperplasia in Mouse Skin Following Single Doses of Ultraviolet Light . . . 97

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